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CHARACTERIZATION OF VIRULENCE IN CLINICAL ISOLATES OF *STREPTOCOCCUS PNEUMONIAE*

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CHARACTERIZATION OF VIRULENCE IN CLINICAL ISOLATES OF STREPTOCOCCUS PNEUMONIAE

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“Pneumococcus is altogether an amazing cell. Tiny in size, simple in structure, frail in make-up, it possesses physiological functions of great variety, performs biochemical feats of extraordinary intricacy and, attacking man, sets up a stormy disease so often fatal that it must be reckoned as one of the foremost causes of human death. Furthermore, living or dead, whole or in part, on entering the animal body Pneumococcus starts a train of impulses, stimulating all the reactions grouped under those inclusive phenomena known as immunity.”

Dr. Benjamin White, 1938 in “The biology of pneumococcus”.

ABSTRACT

Streptococcus pneumoniae is a common colonizer of the nasopharynx of healthy humans, especially children. The pneumococcus is also a major cause of morbidity and mortality, giving rise to diseases such as pneumonia, septicemia and meningitis. The aim of this thesis was to characterize clinical pneumococcal isolates, and thereby increase our understanding of commonalities and differences in how disease develops in infections caused by different pneumococcal strains.

The immune system together with competing pneumococcal strains and other bacterial species, impose a selective pressure on the pneumococcus that leads to the expansion of certain lineages and the remission of others. Our results show that the invasive disease potential varies between serotypes, and also between sequence types (STs) determined by Multi-Locus Sequence Typing (MLST). Furthermore, using Pulsed-Field Gel Electrophoresis (PFGE) we found a divergence of lineages within MLST clones, which was associated with differences in their potential to cause invasive disease. These PFGE clones show intraclonal variation in virulence factors, and in the ability to recruit factor H, a host complement factor utilized by the bacterium to evade the immune system.

Selection of pneumococcal variants occurs both during colonization and disease. We show that during invasive disease there was a selection for serotype 1 variants with impaired hydrogen peroxide production due to mutations in the pyruvate oxidase gene *spxB*. These variants showed greater virulence *in vivo* and were more resistant to clearance by macrophages. An examination of patient samples revealed that *spxB* mutants were present during invasive disease in humans as well.

Variations between pneumococcal strains also affect tissue tropisms during infection, as some serotypes are associated with pneumonia and others with invasive disease. Using two virulent strains of serotypes 2 and 3 we show that the manifestations of disease in mice differed. Infections caused by serotype 3 were primarily confined to the lungs, and the bacteria were resilient to opsonization and clearance by macrophages. In contrast, infection with serotype 2 caused an invasive infection, which spread rapidly from the lungs to the blood.

The genome of the pneumococcus, including several of the bacterial strains in this thesis, has been shaped by the incorporation of lysogenic bacteriophages. We show that a bacteriophage contributed to the virulence during invasive disease of a serotype 1 strain, a serotype with high invasiveness. In the absence of the phage encoded *pblB* gene, bacteria were cleared from the blood.

The results presented in this thesis highlight the genetic and functional variability of pneumococcal strains within and between serotypes, and show that this variability influences the ability of individual strains to cause disease.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals throughout this thesis:

I. BROWALL, S., **NORMAN**, **M**., TANGROT, J., GALANIS, I., SJOSTROM, K., DAGERHAMN, J., HELLBERG, C., PATHAK, A., SPADAFINA, T., SANDGREN, A., BATTIG, P., FRANZEN, O., ANDERSSON, B., ORTQVIST, A., NORMARK, S., HENRIQUES-NORMARK, B. 2014. Intraclonal variations among Streptococcus pneumoniae isolates influence the likelihood of invasive disease in children. *The Journal of infectious diseases,* 209**,** 377-88.

II. SYK, A.*****, **NORMAN**, **M**.*****, FERNEBRO, J., GALLOTTA, M., FARMAND, S., SANDGREN, A., NORMARK, S., HENRIQUES-NORMARK, B. 2014. Emergence of hypervirulent mutants resistant to early clearance during systemic serotype 1 pneumococcal infection in mice and humans. *The Journal of infectious diseases,* 210**,** 4-13.

III. **NORMAN**, **M**., GALLOTTA, M., NANNAPANENI, P., SENDER, V., PATHAK, A., BOOTSMA, H., BROWALL, S., JONCZYK, M., HASTE, L., HERMANS, P., ANDREW, P., HENRIQUES-NORMARK, B.
Streptococcus pneumoniae strains with similar mortality show different patterns of disease progression in mice.
Manuscript

IV. **NORMAN**, **M**., SYK, A., BROWALL, S., HENRIQUES-NORMARK, B.
A bacteriophage contributes to virulence of *Streptococcus pneumoniae* serotype 1 during invasive disease.
Manuscript

***** The authors contributed equally to the work.

CONTENTS

LIST OF ABBREVIATIONS

CAP	Community acquired pneumonia
Cbp	Choline binding protein
CC	Clonal complex
CFU	Colony forming units
CNS	Central nervous system
Cps	Capsule
CR	Complement receptor
CRP	C-reactive protein
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
FACS	Fluorescence activated cell sorting
H_2O_2	Hydrogen peroxide
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPD	Invasive pneumococcal disease
IRAK	IL-1 receptor-associated kinase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAC	Membrane attack complex
MARCO	Macrophage receptor with collagenous structure
MASP	MBL-associated serine protease
MBL	Mannose binding lectin
MHC	Major histocompatibility complex
MLST	Multi-locus sequence typing
MOI	Multiplicity of infection (i.e. the number of bacteria per host cell)
MyD88	Myeloid differentiation primary-response protein 88
NET	Neutrophil extracellular trap
NLRP3	Nod-like receptor protein 3

NOD	Nucleotide-binding oligomerization domain
NOS	Nitric oxide species
OR	Odds-ratio
PAFr	Platelet-activating factor receptor
Pbl	Platelet-binding locus
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugated vaccine, variants cover 7, 10 or 13 serotypes
PFGE	Pulsed-field gel electrophoresis
pIGr	Poly-immunoglobulin receptor
PPV	Pneumococcal polysaccharide vaccine
PRR	Pattern recognition receptor
Psp	Pneumococcal surface protein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SIGN	Specific intracellular adhesion molecule-grabbing non-integrin
SIGN-R1	SIGN related-1
SR	Scavenger receptor
SR-A	Scavenger receptor A
ST	Sequence type, determined by MLST
TLR	Toll-like receptor
TNF	Tumor necrosis factor
WHO	World Health Organization
WTA	Wall teichoic acid

1 INTRODUCTION

Streptococcus pneumoniae, also known as the pneumococcus, is a facultative anaerobic Gram-positive lancet-shaped coccoid bacterium. It typically grows in pairs as diplococci or in short chains. The surface of the pneumococcus is coated with a polysaccharide capsule (Figure 1), but non-typeable variants without capsule also exist.

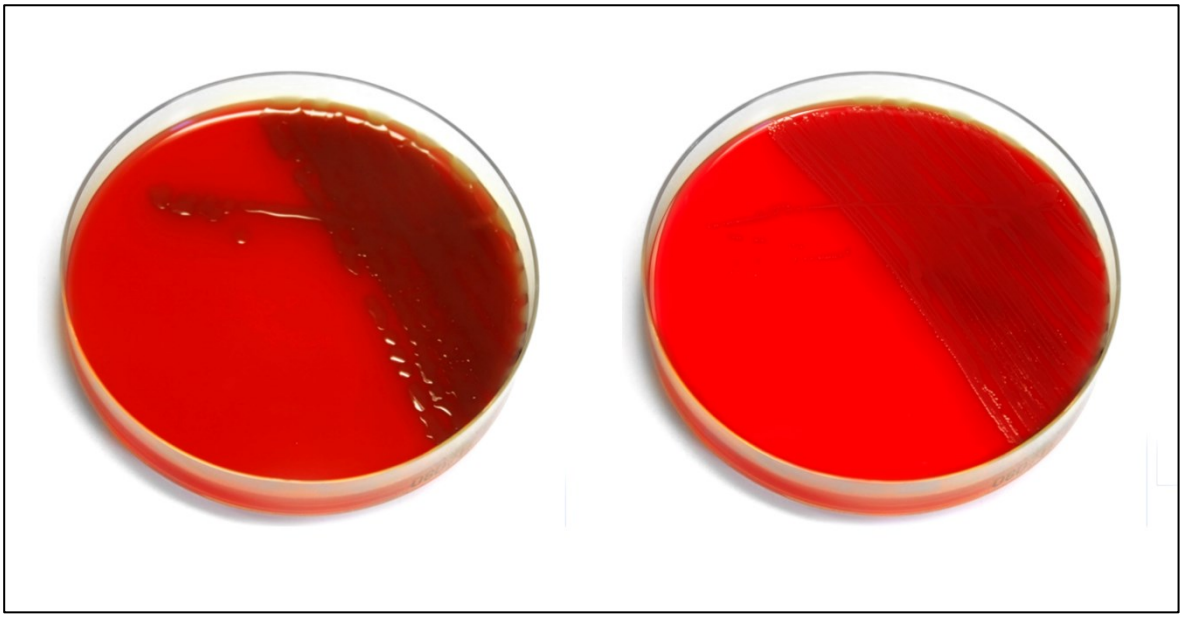

Figure 1. *Streptococcus pneumoniae* forms mucoid colonies due to the production of capsule, as illustrated by the encapsulated serotype 3 strain (left) in contrast to the non-encapsulated R6 strain (right).

The pneumococcus was first isolated in 1880 independently by George Miller Sternberg, from a rabbit that he had previously been injected with his own saliva, and from the saliva of a rabies-infected patient by Louis Pasteur [1].

Historically the pneumococcus has been a devastating pathogen. It is believed that bacterial pneumonia after preceding influenza infection was the major cause of mortality during the 1918-1919 Spanish influenza pandemic, which claimed the lives of an estimated 50 million people [2, 3]. The pneumococcus was a major etiological cause for these bacterial superinfections [3]. Even though the Spanish influenza was an extraordinary pandemic, pneumococcal infections were in themselves severe. Bacteremic pneumococcal pneumonia was associated with a mortality of 80% without antimicrobial treatment [4]. The first therapeutic option for pneumococcal infections was serum therapy which was used during the 1930s, and reduced the mortality to 45%. During the 1950s, after the introduction of penicillin, the mortality was down to 17% for bacteremic pneumococcal pneumonia [4].

1.1 THE PNEUMOCOCCAL GENOME AND ITS PLASTICITY

In 2001 the first completely sequenced genomes, of the TIGR4 and R6 strains, of *S. pneumoniae* were published [5, 6]. The two genomes were comprised of 2-2.2 million base pairs (Mbp) with 2,100-2,200 genes and a G+C content of around 40%.

The genome of *S. pneumoniae* is highly variable between strains due to the natural transformability of the bacterium, which allows the bacterium to take up single-stranded DNA and incorporate it into its own genome. The phenomenon of natural transformation was first observed in relation to the phenomenon of capsular switching by Griffith in 1928 [7]. The principle was extensively studied by Avery *et al*. in 1944, whom showed that the transforming material was an entity different from proteins and suggested it to be DNA [8].

The core genome is shared between pneumococcal strains and constitutes around two-thirds of the total gene content whereas the remaining third of the genes constitute accessory regions, defined as a stretch of at least 3 genes absent in at least one strain [9, 10]. To a large extent the accessory genes, which include the capsule locus, shape the virulence of pneumococcal strains.

The primary site of exchange of genetic material between pneumococcal strains is believed to be in the nasopharynx of the host. In a case study, one infant was found to carry multiple strains of pneumococci during a seven month period, and it was estimated that 8% of the genome was exchanged. The size of the genetic exchanges varied between 0.4 and 235 kb, with an average size of 28 kb [11]. Transformation frequencies vary greatly between pneumococcal strains, with some being virtually non-transformable *in vitro* [12].

1.2 PNEUMOCOCCAL DISEASE

Pneumococcal infections are a major cause of morbidity and mortality world-wide especially among children, where it is the most common cause of bacterial pneumonia [13-15]. In 2005 it was estimated that 1.6 million people die annually of pneumococcal infections, 700,000-1,000,000 of these deaths were children under the age of five [16]. Pneumococcal infections can manifest in several different ways including mild mucosal infections, such as otitis media and sinusitis, or more severe diseases such as pneumonia, septicemia and meningitis. The burden of disease is not evenly spread across the globe. It has been estimated that approximately 60% of all pneumococcal deaths in children under the age of five occur in ten countries in Africa and Asia, and 90% of these deaths are caused by pneumonia (Figure 2).

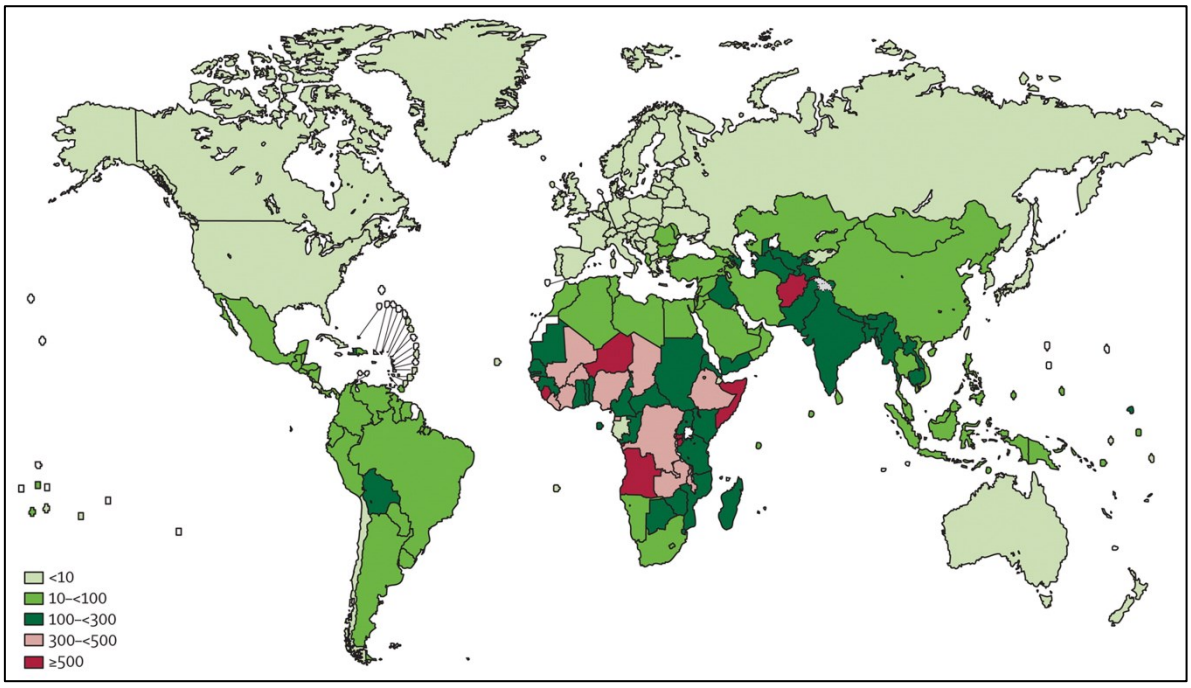

Figure 2. Mortality rate due to pneumococcal disease. Estimates are presented as per 100,000 children younger than 5 years of age. Adopted from [17].

1.2.1.1 Pneumococcal carriage

Pneumococcal infections are transmitted between people by droplets or aerosols. Successful transmission results in colonization of the nasopharynx, which in the vast majority of cases does not lead to the development of disease. Approximately 60% of children attending day-care centers are asymptomatically colonized, but lower colonization rates are found among older children. In the adult population around 5% are carriers of pneumococci [18-21].

Carriage rates of pneumococci in infancy and early childhood vary greatly between different geographical locations largely due to socioeconomic factors. Among infants in the Gambia 77% were colonized by two months of age and 80% were colonized at 12 months of age but with a turnover in serotypes [22]. In Finland less than 10% of children were carriers of pneumococci at 2 months, and during the first year 56% of children had one carriage episode [23, 24]. Similar results were reported from Israel where 42% of infants were colonized during their first year of life, and colonization peaked between two to three years of age with 69% of children being colonized [21].

It is possible to carry more than one serotype at a time, but the frequency of carriage of multiple serotypes depends on the prevalence of carriage. In a study from Malawi in children up to 13 years of age, 27% carried two serotypes and 13% carried three or four serotypes [25]. A smaller study from the US revealed that 5% of children in two day-care centers carried multiple genetic variants [26].

1.2.1.2 Manifestations of pneumococcal disease

Colonization is regarded as a prerequisite for the development of disease. In order for disease to develop the pneumococcus must be able to spread to the middle ear, sinuses or further down in the respiratory tract to the bronchi and alveoli. Invasive pneumococcal disease (IPD) occurs if the pneumococcus gains access to the bloodstream, which can lead to septicemia. Another form of invasive disease, meningitis results if the bacterium is able to transverse the blood-brain barrier and invades the central nervous system.

Acute otitis media (AOM) is a common manifestation of pneumococcal infections. Repeated episodes of AOM or complicated infections can result in hearing loss. AOM exerts a heavy burden on health services as it is the most common infection that requires antibiotic treatment in children under the age of five, with approximately 3.1 million reported cases per year in the US [27]. The high prescription of antibiotics to treat otitis media is a cause of concern, as it can lead to increased antibiotic resistance. It has therefore been suggested that antibiotic treatment may not be justified in milder cases of AOM, as the benefit of treatment is minor in terms of resolution [28, 29]. However, antibiotic treatment does limit the infection and prevent complications, therefore antibiotics are frequently prescribed [30]. Recurrent infections of the middle ear have been suggested to affect the development and understanding of speech in early childhood [31].

S. pneumoniae is the most common cause of community acquired pneumonia (CAP) causing 30-50% of all cases [32, 33]. WHO estimates that the mortality due to pneumococcal pneumonia is between 10-20% [34], but estimates of mortality tend to vary greatly between studies even when conducted within Europe (1-48%). What is clear, however, is that the mortality is associated with increased age [33, 35]. A study in the UK of patients admitted to the hospital with CAP showed a 30-day mortality of ~5% in those under the age of 65, but a mortality of nearly 50% for patients older than 85 years [36].

Approximately one in four pneumonia cases lead to bacteremia [37], which in turn can result in septicemia caused by a systemic inflammatory response. The diagnosis of septicemia requires a proved or suspected infection as well as clinical symptoms. Septicemic patients can exhibit signs such as fever or hyperthermia, increased heart rate, signs of inflammation, hypotension, organ dysfunction or poor tissue perfusion [38]. The mortality of pneumococcal septicemia is around 20% [39, 40]. An increased mortality has been reported for older patients, people with comorbidities and among male patients [39].

Meningitis is the most severe of pneumococcal manifestations with mortalities around 25-30% [41, 42]. Symptoms of bacterial meningitis include headache, fever, stiffness of the neck and cognitive changes [43]. The host inflammatory response is central for the development of neuronal damage and sequelae, which includes hearing loss, blindness, cognitive impairment and paralysis [44]. Pneumococcal meningitis often results in neurological sequelae, which is

present in 30-50% of survivors [41, 42, 45]. The incidence of pneumococcal meningitis varies across the globe, and children are at higher risk. For children under the age of five the global incidence rate is estimated to be 17 per 100,000 people, with a range of 6-21 and the highest rates found in Africa [17]. However, these are estimates for large regions; they may not accurately reflect the situation in a particular country. In Europe an Austrian study conducted between 2001 and 2008 reported the incidence to be 2.3 per 100,000 people under the age of five [46]. In Sweden 1-2 cases of pneumococcal meningitis in children younger than five were reported annually in 2012 and 2013, but a peak was noted for 2014 with eight cases [47].

1.3 EPIDEMIOLOGY OF STREPTOCOCCUS PNEUMONIAE

Pneumococci are divided into serotypes based on the antigenic properties of the capsule. Currently 97 different serotypes have been described [48]. A pneumococcal strain can exchange its capsule for a capsule of another serotype, so serotypes do not always follow the genetic lineage. Two methods, multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE), allow for genetic typing of strains and are described in the methods section of this thesis.

1.3.1.1 Serotype 1

Serotype 1 together with serotypes 2, 3 and 5 are considered epidemic serotypes as they have been reported to cause outbreaks of pneumococcal disease [49]. The carriage rate of serotype 1 is low, i.e. the serotype is rarely found to colonize healthy individuals [50-53]. Several reports have suggested that the duration of carriage is short with serotype 1 [54-56]. However, serotype 1 cause a significant proportion of IPD and is therefore generally considered highly invasive [50, 51, 57].

Brueggemann and Spratt reported differences in the geographic distribution of serotype 1 lineages. They defined a lineage to include isolates that shared at least four MLST alleles. They found lineage A to dominate in Europe and North America, with the sequence type (ST) ST306 predominately found in Europe and ST227 predominantly found in North America. In Africa lineage B was more frequent among the isolates, this lineage included ST217 [58]. In North America serotype 1 is relatively uncommon but in the rest of the world it caused more than 6% of all cases of IPD before the introduction of vaccination. However there was great geographical variation in incidence between countries. For India a high percentage of IPD was reported to be caused by serotype 1, with up to 10-20% of cases in children younger than 18 years and over 30% of the cases in the adults [59]. The high prevalence of serotype 1 in IPD in India has also been reported in a multicenter study carried out between 1993 and 1997. Serotype 1 was responsible for up to 25% of the cases of pneumococcal disease, with an even higher percentage (31%) of this serotype in isolates from

patients older than five years of age [60]. Serotype 1 has an increased ability to cause disease in healthy adults compared to other pneumococcal serotypes [49, 61].

During the period from 2002 to 2005 an outbreak of meningitis was reported in Burkina Faso. 249 diagnosed cases of pneumococcal meningitis were reported, of which 44% were of serotype 1 [62]. Half of the affected people were older than five years, the mortality did not vary between age groups and the average fatality rate was 46%. A similar mortality (44%) was seen during an outbreak in Northern Ghana between 2000 and 2003, preceding that in Burkina Faso [63]. Serotype 1 was responsible for three out of four of the 117 diagnosed cases of pneumococcal meningitis reported in Ghana between 1998 and 2003. The dominant lineage in this outbreak was ST217. Papers II and IV include experiments performed with strains of serotype 1.

1.3.1.2 Serotype 2

Serotype 2 is one of the most well studied serotypes as it includes the D39 strain that is commonly used in research. However, a peculiarity of this strain is that it used to be a common cause of pneumococcal disease in Europe and North America but is now virtually never isolated [49, 64]. Historical records show that serotype 2 was considered to give rise to severe and invasive infections. In a study from New York (USA) of 202 serotype 2 cases during 1920-1931, 51% of the patients developed bacteremia. This can be compared to 30% of bacteremia in patients infected with serotype 1, which currently is regarded as an invasive serotype. The mortality reported in the study was 49% for patients infected with serotype 2 and 28% for patients infected with serotype 1 [65].

In a retrospective study of IPD spanning 1938-2007 in Denmark, serotype 2 was isolated from approximately 2.5-10% of all IPD cases in 1940-1960. Serotype 2 went into decline during the 1970s, and since then virtually no cases have been reported [49]. In a vaccine trial carried out in adults in South Africa in 1973-1976 serotype 2 was the most prevalent etiological agent with 24 cases of the 103 recorded in the non-vaccinated control group [66]. In the mid-1980s and mid-1990s a prevalence of 1-3% was reported for serotype 2 among South African adults with pneumococcal disease [67].

It would be wrong to assume that serotype 2 is no longer a threat. As most epidemiological surveys are carried out in the developed world there is little information on the epidemiology of serotypes prevalent in other parts of the world. The information available on the epidemiology of serotype 2 is scarce, but cases of serotype 2 are occasionally reported outside of Europe and North America. In a report from 1991-1993 in Papua New Guinea on 13 isolates from pneumococcal bacteremia in infants less than three months old, three were of serotype 2 [68]. Out of 106 isolates collected from patients younger than 15 years old with IPD in Mali between 2002 and 2003 serotype 2 was identified in 14% of the isolates [69, 70].

In the period of 1993 to 1997 the serotype was found in two out of 101 isolates collected across India from children under the age of five with pneumococcal disease [60]. From 2004 to 2007 two out of 45 cases of pneumococcal disease in children under the age of five reported from Nepal were of serotype 2 [71].

In a hospital based survey between 1993 and 1997 from Dhaka (Bangladesh), serotype 2 was identified in ~3% of the 362 isolates from children under age of five with pneumococcal disease [72]. From 2001 to 2009 the situation changed drastically in this age group as this serotype 2 accounted for 20% of the 211 cases of childhood meningitis reported from seven hospitals in Bangladesh. Only one out of 45 serotype 2 isolates was found in the 112 patients who presented with IPD but without meningitis [73].

Serotype 2 predominantly infected children during the first year of life and the median age of infection was significantly lower (three months) compared to that of all other pneumococcal serotypes which caused meningitis (seven months) [73]. Although generally considered a minor serotype, the outbreak of meningitis in Bangladesh as well as historical records show that serotype 2 should not be discounted and may have the potential to reemerge. The D39 strain of serotype 2 was used in experiments presented in paper III.

1.3.1.3 Serotype 3

In a study from the Gambia serotype 3 was the most prevalent serotype isolated from the nasopharynx in ~9% of unvaccinated healthy people in a cross-sectional survey [50]. This serotype is often a cause of pneumonia in patients with comorbidities and also in the elderly [74]. Serotype 3 was characterized as having a low invasive disease potential in a metaanalysis by Brueggemann *et al.* [75]. However, serotype 3 is associated with high mortality in cases of pneumonia [57, 61, 76, 77]. Serotype 3 has also been associated with severe IPD in patients with comorbidities, and with a high risk of developing septic shock [61].

Serotype 3 was associated with necrotizing pneumonia in a study of 124 children under the age of 18 with pneumonia in Utah (USA) between 1997 and 2006. This manifestation was present in 11 out of 14 cases caused by serotype 3. Necrotizing pneumonia was 15 times more likely as a manifestation for this serotypes compared to other pneumococcal serotypes in the study [78, 79]. In line with this report serotype 3 was isolated from 10 out of 13 cases with bronchopulmonary fistulas in children [80]. In a study carried out in Texas (USA) which included patients of all ages, serotype 3 was the only pneumococcal serotype found to be significantly associated with necrotizing pneumonia, and accounted for five of 16 cases [81]. Serotype 3 was also the most frequently isolated serotype from COPD patients with bacteremic and non-bacteremic pneumonia [82]. Experiments in paper III included a strain of serotype 3.

1.3.1.4 Serotype 6B

In unvaccinated populations serotype 6B frequently ranks amongst the five most common serotypes isolated from carriage and disease [83-85]. Although the serotype is regarded as having low invasiveness, pneumonia caused by serotype 6B is associated with a high relative risk of death [51, 75, 77].

In a case study, an six year old patient presented with seven episodes of pneumococcal pneumonia. Serotype 6B was isolated from five episodes and serotype 6A from the subsequent two episodes, both serotypes belonged to the same sequence type [86]. Whole genome sequencing revealed a clonal origin of the isolates with a total of 10 SNPs between the isolates, some of which were potential compensatory mutations to the therapeutics used during treatment. The recurrent infections seem to have been caused by the same 6B strain which had acquired the 6A capsule via horizontal gene transfer in between the disease episodes [86]. Paper I includes experiments with several strains of serotype 6B.

1.3.1.5 Duration of pneumococcal carriage

Several longitudinal studies have estimated the duration of pneumococcal carriage, but the estimated duration varies between serotypes and studies [55, 87, 88]. A study in Kenya with children aged between three months and five years included all serotypes relevant for this thesis, with the exception of serotype 2. In the study the average duration of carriage was estimated to be approximately 30 days. Serotype 6A had the longest carriage duration (50 days) and the corresponding numbers for serotypes 6B, 3 and 1 were 46, 11 and 9 days [55].

1.3.1.6 Experimental human carriage studies

In experimental human carriage studies with pneumococci, volunteers are administered a bacterial suspension into the nose. The approach was first used to investigate the immunogenicity of pneumococcal antigens [89, 90]. Since then experimental human carriage has also been used to investigate the dynamics of acquisition and clearance during colonization with the *S. pneumoniae* 6B strain BHN418 [91]. The results from the model show that the rate of acquisition is dose dependent and that a previous colonization protects against a subsequent colonization event with the same strain [92]. Volunteers that had oropharyngeal swabs positive for asymptomatic viral infection five days before inoculation with bacteria were more prone to acquire pneumococcal colonization [93]. Another study using the model showed that the levels of anti-inflammatory transforming growth factor β (TGFβ) or immunomodulatory interleukin 10 (IL-10) were positively correlated with establishment of carriage [94].

1.4 THERAPEUTIC OPTIONS

β-lactam antibiotics, primarily penicillin, and macrolides, such as erythromycin, are often used to treat infections caused by *S. pneumoniae*.

The β-lactams bind to penicillin-binding proteins (PBPs), which are involved in the synthesis of the bacterial cell wall and thereby inhibit their function. Inhibition of PBPs leads to the inability of the bacterium to form and crosslink peptidoglycan chains and triggers autolysis [95]. Resistance to β-lactams in *S. pneumoniae* results from mutations in the PBPs, giving the binding site reduced affinity for the antibiotic [96]. The level of penicillin tolerance and resistance varies greatly between regions with figures in excess of 50% of isolates from Southeast Asia reported as being non-susceptible to penicillin [97]. In 2013 6.8% of invasive pneumococcal isolates in Sweden were non-susceptible to penicillin. The rates of non-susceptibility to penicillin varied between 1 to 30% in the countries participating in the European Centre for Disease Prevention and Control (ECDC) surveillance program in 2013 [98].

Macrolides such as erythromycin are bacteriostatic and inhibit bacterial replication by binding to the 23S ribosomal RNA of the 50S ribosomal subunit, thereby blocking the elongation of the polypeptide. Macrolides are used as alternatives for patients whom are allergic to penicillin [95]. Resistance can be developed by methylation of the macrolide target site by the *ermB* methylase, leading to high-level resistance. Low- to mid-level resistance can be conferred by the *mefA* gene encoding an efflux pump actively transporting out the drug [99]. In 2013 ECDC reported between 1.5 and 38.1% of isolates to be non-susceptible to macrolides, for Sweden 6.5% of isolates were reported as non-susceptible [98].

Isolates which are show decreased susceptibility to both penicillins and macrolides are a major concern. For 2013 ECDC reported that the percentage of isolates that were non-susceptible to both classes of antibiotics was 3.2% in Sweden and varied between 0-27% in Europe [98]. For pneumococcal infections which are difficult to treat with other antibiotics vancomycin is considered to be the last resort. Vancomycin belongs to the antibiotic class of glycopeptides. The mechanism of action is to disrupt cell wall synthesis by binding to D-alanine-D-alanine thereby preventing the formation of cross links between peptidoglycan strands. It is mainly used in severe systemic infections [95]. Tolerance can develop by changes in the two-component system VncS-VncR [100], but the mechanism is not understood.

1.5 RISK FACTORS

Apart from age, factors such as ethnicity and socioeconomic status influence the risk of acquiring a pneumococcal infection. Several chronic conditions including heart and lung disease, splenic dysfunction and diabetes increase the risk of pneumococcal infections [101-

103]. Malignancies and immunosuppression (including patients infected with HIV and recipients of organ transplants) are also risk factors for pneumococcal infection. Risk factors related to life-style include substance abuse such as alcohol abuse and smoking [101, 102].

1.6 VACCINE

As pneumococci are a considerable cause of mortality and exerts a heavy toll on health-care budgets globally there is a need for effective vaccines and implementation of vaccination strategies to prevent pneumonia and IPD. The need to prevent pneumococcal disease is underlined by the increase of pneumococcal strains with reduced susceptibility to antibiotics.

Currently there are two types of vaccines available on the market, the pneumococcal polysaccharide vaccine (PPV) containing 23 capsular polysaccharides, and the pneumococcal conjugated vaccines (PCV) where 7, 10 or 13 capsular polysaccharides have been conjugated to a protein carrier to increase the immunogenicity. The most frequently used pneumococcal vaccines are listed in Table 1.

Table 1. Serotype covered by the pneumococcal conjugated vaccines (PCV) and the pneumococcal polysaccharide vaccine (PPV) [102, 104]. Serotypes added in PCVs with higher valency are indicated in bold.

Vaccine	Manufacturer	Serotypes covered by the vaccine
PCV7 - Prevnar®	Pfizer	4, 6B, 9V, 14, 18C, 19F, 32F
PCV10 - Synflorix™	GSK Biologicals	**1**, 4, **5**, 6B, **7F**, 9V, 14, 18C, 19F, 23F
PCV13 - Prevenar ™	Pfizer	1, **3**, 4, 5, **6A**, 6B, 7F, 9V, 14, 18C, **19A**, 19F, 23F
PPV23 - Pneumovax	Merck	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F

PPV23 elicits a T lymphocyte (T cell) independent immune response by activating B lymphocytes directly, which give a poorer immunological memory than the PCVs [105]. Studies of the immunogenicity of the vaccines have shown that PCV13 is able to elicit an immune response comparable or superior to PPV23 in adults one month following vaccination [105-107]. One possible exception was noted for serotype 3 [107]. One year after vaccination the antibody titers for individuals vaccinated with PCV13 were higher than for those vaccinated with PPV23 [105, 106, 108].

PCV has been included in the childhood vaccination program in Sweden since 2009. In 2010 PCV10 and PCV13 replaced PCV7 in the vaccination program. The doses are administered at 3, 5 and 12 months of age. Approximately 42% of the children vaccinated in Sweden receive PCV10 and 58% PCV13, and more than 97% of children are administered a complete set of three doses. Before PCV was introduced the incidence of IPD was 36.9 per 100,000 children under the age of 2 years old (reference year 2006). Following introduction the incidence has decreased to 10.8 in 2014. For children under 5 years of age a decrease from 19.3 per 100,000 to 5.8 was noted for the same period [47]. In Sweden vaccination with PPV23 is

recommended for people aged over 65 years, and for individuals over 2 years of age in risk groups [109].

Several meta-analyses have shown an effect of vaccination in terms of a decrease in carriage and IPD, caused by vaccine serotypes in vaccinated populations [110-114]. These studies have primarily been carried out in developed countries. Several studies have also shown a concomitant decrease of PCV serotypes in disease amongst unvaccinated people, suggesting a herd immunity effect [110, 115]. However, the effect of vaccination has been hampered in some studies by a concomitant increase in disease caused by serotypes not covered by the vaccines. There is concern that the increased use of vaccination leads to serotype replacement, as serotypes covered by the vaccine are selected against and replaced by other serotypes [49, 52, 115-117]. It therefore remains to be seen to what extent vaccination with PCV will result in a long-term reduction in severe pneumococcal disease.

1.7 THE IMMUNE SYSTEM

1.7.1 How the immune system keeps us healthy – an overview

The immune system includes physiological barriers and structures, cells and molecules that are involved in keeping us healthy by protecting us from potential pathogens, clearing away particles and eliminating non-functional or cancerous cells. However, a poorly adapted immune response can create severe pathology in its attempt to fight an infection. By convention the immune system is divided into the innate and the adaptive immune system.

Innate immunity is poised to act upon the first encounter with a pathogen, and gives rise to the classical signs of inflammation *rubor* (redness), *calor* (heat), *tumor* (swelling) and *dolor* (pain). The innate defense is either already in place or quick to respond to an infection. The innate response has the ability to recognize a broad range of pathogenic microorganisms, and to trigger activation of both the innate and adaptive arms of the immune system.

For a respiratory tract pathogen the first part of the immune system the bacterium is likely to encounter are tissue barriers, mucus and the beating of cilia that serve to impede microbes and to contain them at sites were they can do limited harm. Antimicrobial peptides present in secretions such as saliva and mucus help to keep the microbes present on mucosal surfaces under control.

The complement system does not include cells as effectors but consists of an elaborate network of different proteins. The interactions are complex and interlinked but serve to detect microbes in order to kill them by assembling the membrane attack complexes (MAC), which form pores in the membranes of pathogens. The complement system also facilitates phagocytosis by coating the surface of microbes with opsonins.

The ability of the innate response to fight bacterial infections caused by Gram-positive bacteria is largely dependent on phagocytic immune cells such as macrophages and neutrophils. Phagocytic immune cells primarily ingest microbes in order to kill them, but can also secrete antimicrobial effectors and signaling molecules such as cytokines. An important role in the activation of the immune system is played by dendritic cells. When dendritic cells phagocytose bacteria, they respond by presenting microbial antigens to T lymphocytes and secreting cytokines to activate adaptive immunity.

Adaptive immunity takes longer to mobilize in response to an infection, but brings with it an incredible diversity and specificity. It has the ability to target a vast repertoire of molecules associated with its microbial target, and does so with high specificity making the response tailored for the pathogen. Immunological memory is one of the key features in the adaptive immune response and allows the system to respond faster, with greater force and with a more qualitative immune response if it encounters the same pathogen again. Antibodies are produced by B lymphocytes and play a key role in the adaptive response against the pneumococcus. B lymphocytes can differentiate into memory cells, which retain the immunological memory should infection with the same pathogen reoccur.

The cross-talk between the different cells of the immune system is to a large extent mediated by a large repertoire of cytokines. Cytokines form the alarm signals triggered by a pathogen and they stimulate immune cells to infiltrate to the site of infection and stimulate the development of new immune cells. Cytokines are vital in linking the different part of the immune system, and for balancing the immune response and returning the tissue to homeostasis once the infection has been cleared. The complex interactions between immune cells and cytokines, are in many contexts, not fully understood.

The papers presented in this thesis describe the early events in the response to a pneumococcal infection. The following section is therefore intentionally focused on the role of phagocytic immune cells and the complement system associated with the innate immune response to pneumococcal infections in order to provide a context for the mechanisms discussed in the papers.

1.7.2 The immune cells

1.7.2.1 Macrophages

As resident cells in tissues throughout the body, including the lungs and respiratory tract, macrophages are among the first responders to a bacterial infection. Macrophages are derived from monocytes released from the bone marrow and differentiate into macrophages in the tissue. In the case of alveolar macrophages the monocytes first differentiate into parenchymal, or interstitial, macrophages within the lung tissue before further differentiation into alveolar macrophages residing in the alveoli of the lung [118, 119]. Both the alveolar and

parenchymal macrophages can undergo proliferation, allowing for the replenishment of the macrophage population during homeostasis [118, 119]. However, during infection an influx of monocytes into the lung helps to reconstitute the numbers of macrophages. The primary role of macrophages is to phagocytose bacteria. Internalization occurs by invagination of the cellular membrane engulfment as the membrane fuse around a microbe, encasing the microbe in a vacuole called a phagosome. The phagosome then fuses with lysosomes and acidifies, generating phagolysosomes [120].

Fc receptors are responsible for phagocytosis of particles opsonized with antibodies. Macrophages express several different types of Fc receptors including the FcγRIIA which is a low affinity receptor that is mainly responsible for binding IgG_2. FcγRI is a high affinity receptor and FcγRIIA and FcγRII are low affinity receptors for IgG_1 [121]. The importance of these receptors in invasive infections has been demonstrated in mice [122]. To allow for the uptake of particles opsonized with components of the complement system macrophages express a set of complement receptors (CRs). The receptor CR1 binds C3b, iC3b and C4b, and the receptors CR3 and CR4 bind the opsonin iC3b. The effects of the CRs are potentiated by secondary signals such as binding immune complexes by Fc receptors. Phagocytosis via CRs does not stimulate ROS production or lead to an inflammatory response [123].

There are different classes of scavenger receptors with scavenger receptor A (SR-A), of class A, implicated in the defense against pneumococcal infections SR-A exists in three isoforms which are found on macrophages and dendritic cells [124]. SR-A mediates phagocytosis of particles and non-opsonized microbes. Mice deficient in SR-A are more susceptible to pneumococcal pneumonia, exhibiting higher mortality and impairment in the ability to control the bacterial numbers in lungs during the first hours after infection [125]. This correlates with observations that alveolar macrophages isolated form SR-A-deficient mice have reduced ability to phagocytose *S. pneumoniae* compared to the wild-type macrophages [125, 126].

The macrophage receptor with collagenous structure (MARCO) is a class A scavenger receptor found in both humans and mice [127, 128]. It is expressed by macrophages in the liver, marginal zone of the spleen, in the medulla of the lymph nodes as well as by alveolar macrophages, and its expression is up regulated in response to a bacterial infection [128-130]. In a study by Arredouani *et al.* mice deficient in MARCO showed impaired ability to clear bacteria from the lungs, and had higher mortality. MARCO-deficient mice also exhibited increased inflammation, as they had higher levels of TNFα and MIP-2 in lavage fluid and with enhanced neutrophil recruitment into the lungs during the first 24 hours of infection [129]. A more recent study supports the importance of MARCO in the clearance of colonizing pneumococci in the upper respiratory tract. Furthermore, MARCO-deficiency was associated with reduced production of pro-inflammatory cytokines and reduced influx of neutrophils [131]. It has been suggested that the role of SR-A in mediating phagocytosis of non-opsonized bacteria by alveolar macrophages is subordinate to that of MARCO [132].

The C-type lectin receptor in humans, dendritic cell-specific intracellular adhesion molecule-grabbing non-integrin (DC-SIGN), has five homologues in mice. Of interest in the context of pneumococcal infection is the homologue receptor SIGN related-1 (SIGN-R1). SIGN-R1 is expressed by the intraperitoneal macrophages, macrophages in the medulla of lymph nodes and marginal zone macrophages in the spleen [133-135]. It has been shown that SIGN-R1 mediates uptake of pneumococci in the spleen [133]. SIGN-R1-deficient mice were more susceptible to intraperitoneal infection with pneumococci [135], and developed more severe pneumonia and bacteremia after intranasal infection [136]. SIGN-R1 can also mediate activation of the complement system in the spleen by assembling the C3 convertase without the involvement of Factor B or antibodies [137].

It is not fully understood how macrophages kill pneumococci once the bacteria have been phagocytosed. It is quite possible that it is a combinatorial effect of different mechanisms which leads to the successful killing of internalized pneumococci. The generation of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide have been suggested as potential mechanisms involved in the killing of bacteria within macrophages [123]. However, pneumococci produce high amounts of hydrogen peroxide and have developed tolerance to the actions of ROS. Furthermore, macrophages generate ROS via their mitochondria which associate with phagosomes [138]. However, this system produces less ROS than neutrophils which use a myeloperoxidase system [123]. ROS has the potential to react with the nitric oxide (NO) produced in response to pneumococci in human and mouse macrophages [139, 140], to form reactive nitrogen species (RNS), which is a more potent molecule for killing pneumococci [141]. The phagosomes of macrophages are also rich in proteases, as well as Nramp1 a scavenger of divalent cations which removes these essential micronutrients from the phagolysosome [123, 142].

Phagocytosis of a large number of bacteria leads to an impaired ability of the macrophage to efficiently kill internalized bacteria. Under such circumstances the macrophage can undergo apoptosis, which leads to killing of bacteria without the release of pro-inflammatory mediators [143-145]. In mice this mode of action has shown to be efficient in reducing disseminated disease [145].

1.7.2.2 Neutrophils

If the response of alveolar macrophages is not sufficient to control a pneumococcal infection in the respiratory tract, neutrophils will start to infiltrate the tissue from the blood in large quantities. Neutrophils are short-lived phagocytic cells that are constantly released from the bone marrow and circulate in the bloodstream. When needed they are recruited to the site of infection by the pro-inflammatory cytokines, such as TNFα, IL-1β, IL-6 and IL-8, released by macrophages and epithelial cells [146]. The role neutrophils play during an infection is not limited to the lungs, as they are also involved in the clearance of bacteria during systemic

infections. Efficient phagocytosis of bacteria by neutrophils is dependent on opsonization of the microbe, which is mediated by Fc-receptors or CR3.

Neutrophils are potent killers of bacteria, and produce large quantities of ROS. Failure to control inflammation and the release of ROS by neutrophils can lead to severe tissue damage. The tissue lesions and consolidation seen as a result of pneumonia is, to a large extent, mediated by overt inflammation caused by neutrophils. As mentioned pneumococci are tolerant to ROS and killing of pneumococci by neutrophils is not dependent on ROS [147]. Instead neutrophils utilize serine proteases, which include proteinase 3, elastase and cathepsin G [147]. These protases are kept in pre-packed granules, which fuse with the phagosome after internalization of bacteria. Neutrophils also have the ability to form neutrophil extracellular traps (NETs) consisting of the neutrophil's own DNA with the associated histones and elastase form the granules. The NETs trap bacteria and also have bactericidal properties against many bacteria. However, the pneumococcus remains viable in NETs, and the capsule offers limited protection against NET entrapment [148, 149]. It is likely that the host uses NETs to confine the pneumococci and thereby limit the spread of the infection. To escape entrapment pneumococci express endonuclease A, which degrades the DNA and allows the bacteria to be released from the NETs [148].

1.7.2.3 Dendritic cells

Dendritic cells (DCs) are antigen presenting cells which serve to sample and detect invading microorganisms and subsequently activate the adaptive immune response. They are strategically positioned along the respiratory tract to sample the mucosa for microbes and microbial fragments. Phagocytosis results in the maturation of the DC, which allows it to express processed antigens on MHC class II molecules. One of the factors pneumococci express to avoid phagocytosis is PavA which has been shown to inhibit uptake of pneumococci by human DCs [150]. Pneumolysin has also been shown to cause apoptosis in DCs and reduced DC activation [151].

DC maturation stimulates expression of co-stimulatory molecules and T cell adhesion molecules, and triggers the migration of the activated DC to the lymph node where they present the antigen to naïve T lymphocytes [152]. DCs play a crucial role to elicit an adaptive immune response by cytokine signaling and the presentation of antigens to T and B lymphocytes. They are therefore crucial for the development of a qualitative antibody response consisting of the IgG subclass, and in the generation of protective immunity after immunization that can generate resistance against subsequent infections [153-155].

1.7.2.4 T lymphocytes

CD4+ Th17 population of T lymphocytes is important for the recruitment of neutrophils and by secreting of IL-17 they stimulate a potent neutrophil response. IL-17 triggers the release of the cytokine and neutrophil attractant IL-8 from lung epithelial cells [156, 157]. IL-17, as

well as IL-6, also stimulates the production of mucin in human primary trachea-bronchial epithelial cells [158]. IL-17 mediated induction of cytokines provides an important signal in the response at the mucosa against infections caused by bacteria, as it reduces dissemination and promotes neutrophil mediated clearance [159]. Release of IL-17 and IL-22 by Th17 cells stimulate the release of β-defensin 2 in human lung epithelium [160]. The release of β-defensin 2 can also be triggered by *S. pneumoniae* alone, and has been shown to have a bactericidal effect *in vitro* [161, 162]. IL-17 has been shown to be important for immunity and the neutrophil mediated clearance of pneumococci during colonization [163]. Killing of activated pulmonary DCs and macrophages by the γδ T cell subset in mice is one of the ways by which the host resolve the state of inflammation as the number of bacteria subsides and the tissue returns to homeostasis [164].

1.7.2.5 B lymphocytes

B lymphocytes are a part of the adaptive immune system but are nonetheless important during the initial response to pneumococci as antibodies facilitate uptake of pneumococci by Fc-receptors and aid in complement activation [122, 165, 166]. Both specific antibodies, induced by a previous encounter with pneumococci, and naturally occurring antibodies targeting common microbial antigens play an important role in the activation of the complement system [122, 167].

The efficacy of vaccines depends on the ability to trigger B lymphocytes to elicit a protective antibody response and immunological memory. Polysaccharides are T cell independent antigens which activates B lymphocytes without the involvement of T lymphocytes. By conjugation of a peptide to the polysaccharide in PCV vaccines, the antigen functions as a T cell dependent antigen [168]. In children younger than 2 years old the spleen has not fully developed and the B lymphocytes have not matured [169]. Young children are therefore unable to produce antibodies in response to T cell independent antigens and hence the PPV does not offer a protective effect [168]. The conjugated PCVs were developed as children of this age are able to respond to T cell dependent antigens. There have been reports that the function of B lymphocytes deteriorates with age, and that elderly people vaccinated with PPV produce antibodies with reduced capacity to opsonize bacteria [170, 171].

1.7.3 The complement system

The complement system is a complex network of plasma proteins that performs several functions; to opsonize microbes, recruit phagocytes and to assemble the MAC to kill bacteria. The system is built on the stepwise activation of components to allow for the initial recognition signal to be amplified through the complement cascade. The complement system needs to be tightly regulated, as activation triggers a cascade of reactions that not only has the potential to kill microbes but also host cells.

There are three activation pathways for the complement system, the classical, the alternative and the lectin pathways. All three pathways are of relevance in the host response to pneumococci. The way the pathogen is recognized varies between the pathways but once the activation has been initiated all pathways lead to the formation of a C3 convertase. The C3 convertase cleaves plasma C3 into C3b. C3b is deposited on the surface of the microbe with the concomitant release of the cleavage product C3a, which acts as a chemoattractant for neutrophils. The C3 convertase can associate with factor C5 and form the C5 convertase to recruit complement protein C6 to C9. These proteins then assemble into the MAC and form a pore to permeabilize the cellular membrane. Gram-positive bacteria are not lysed by the MAC due to their cell wall which prohibits the assembly of the MAC subunits required for formation of the pore. The primary role of the complement system in the context of a pneumococcal infection is therefore to opsonize bacteria to allow for more efficient phagocytosis by neutrophils and macrophages.

1.7.3.1 Pathways of complement activation

The classical pathway is activated by antibody binding. For this reason it is the only pathway which can be activated by the adaptive immune response. Due to the presence of natural antibodies it is also important during the innate immune response to infections. Natural antibodies are present before an infection and recognize common microbial epitopes, such as the phosphorylcholine residues on the teichoic acids of pneumococci. These antibodies are in general of the IgM isotype.

Activation of the classical pathway occurs when the hexameric C1q molecule is able to bind two or more Fc regions present on antibodies. This ensures that only antibodies bound to an antigen can activate the complement cascade. C1q-binding activates the protease C1r which cleaves and activates the protease C1s that in turns cleaves C4 and C2. The C4b and C2a fragments form the C3 convertase of the classical pathway [172].

In pneumococcal infections the classical pathway can be activated without the involvement of antibodies via the SIGN-R1 receptor primarily, expressed by marginal zone macrophages in the spleen. SIGN-R1 is capable of binding to both the polysaccharide capsule of the pneumococcus and to the C1q protein thereby triggering the activation of the classical pathway, and the assembly of the C3 convertase [137].

The alternative activation pathway is initiated by spontaneous hydrolysis of C3 into C3a and C3b in the plasma (schematic representation in Figure 3). C3b is then deposited on cellular surfaces. Factor H is bound to the surface of host cells and functions as a cofactor for Factor I mediated proteolysis of C3b into iC3b. Factor H also dissociates the C3 convertase of the alternative pathway. Although iC3b still function as an opsonin, it can no longer function in forming the C3 convertase. *S. pneumoniae* is able to utilize the same system by binding factor H via its surface protein PspC, thereby limiting opsonization [172].

The lectin pathway is initiated by the binding of Mannose binning lectin (MBL) or ficolins to the surface of bacteria. These molecules target conserved bacterial motifs, MBL binds mannose residues on polysaccharides and ficolins bind to *N*-acetylglucosamine on glycan strands. This binding leads to the recruitment of MBL-associated serine proteases (MASPs), which activate C4 and C2 [173].

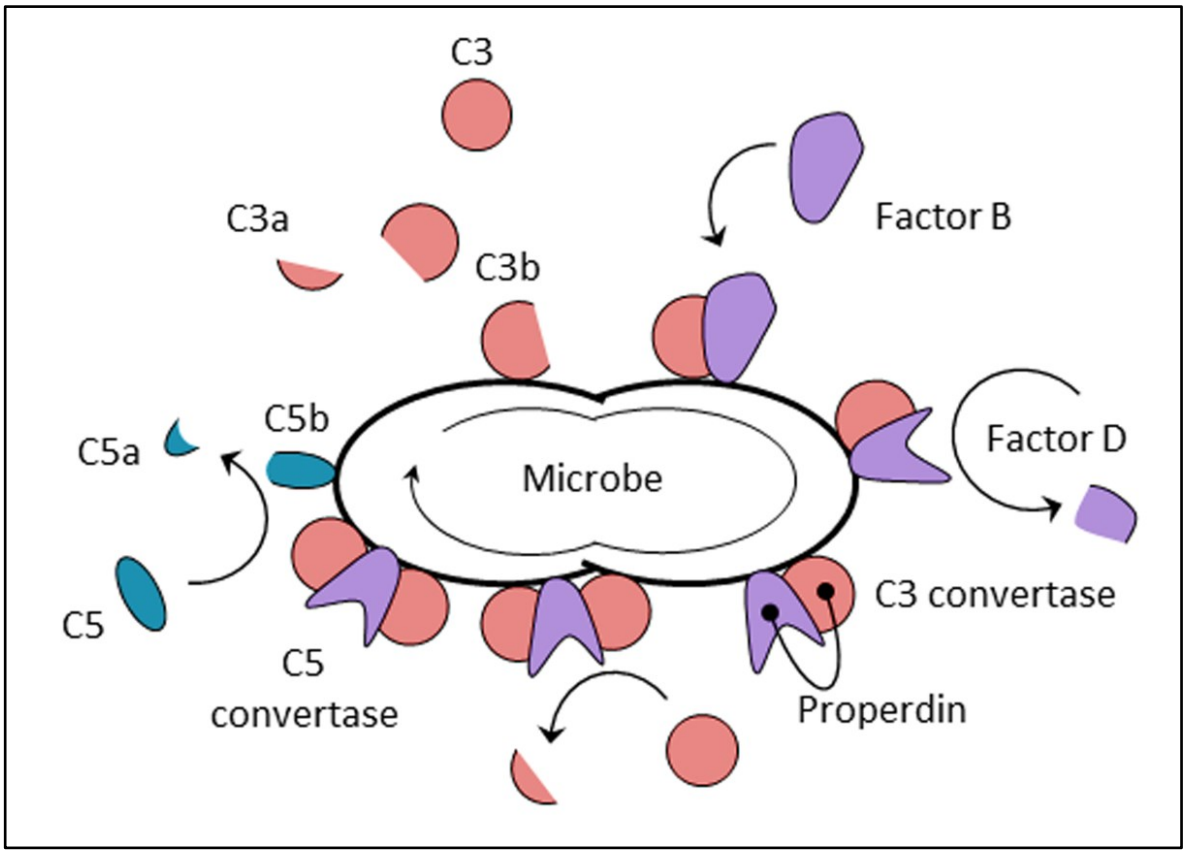

Figure 3. Schematic representation of the complement activation by the alternative pathway as described by Abbas *et al*. [172]. C3 hydrolyses spontaneously in plasma, forming the chemoattractant C3a and allowing the opsonin C3b to bind to the surface of microbes. Factor B binds to the exposed binding site on C3b. Factor B is cleaved by factor D generating the Bb fragment which remains attached to C3b. The formed C3bBb complex is the C3 convertase for the alternative pathway. The C3 convertase is stabilized by properdin, and more deposition of C3b ensues. By binding two C3b molecules per Bb-fragment the C5 convertase is formed. C5 convertase cleaves C5 into C5a and C5b, C5a is an anaphylatoxin and C5b is required for the subsequent assembly of the membrane attack complex (not shown).

1.7.3.2 C-reactive protein and complement activation

C-reactive protein (CRP) is an acute phase protein which is released from the liver in response to an infection. Invasive bacterial disease can lead to a 1,000-fold increase in the amounts of circulating CRP, to levels of 350 mg/L [174]. CRP binds to the choline residues on the lipoteichoic acids of the pneumococcal cell wall [175]. As the binding site for phosphorylcholine on CRP is separate from that of C1q [176], it is possible for CRP to activate the classical pathway in humans.

C-reactive protein is expressed at low levels in mice (2-3 µg/mL during the acute phase) [122]. Mice infected with human C-reactive protein showed increased survival in systemic infections, and increased complement deposition [122, 177, 178]. The protection was dependent on complement but not on Fcγ-receptors [122], but did not require CRP binding to the cell wall of the pneumococcus [178]. The administration of CRP must be concomitant to the bacterial infection, as administration of CRP 36 hours post infection did not affect survival or the progression of disease [178]. C1q in mice are not able to bind to human CRP *in vitro* and therefore seems unlikely to activate the classical pathway in mice [177]. It thus seems that CRP increases complement deposition but does not play a role in the activation of the classical pathway in mice.

1.7.3.3 The complement system in the respiratory tract

Mice deficient in the classical pathway (*C1qa*$^{-/-}$), and for factor B had more had more rapid progression of disease following intranasal infection [166]. IgM deficient mice (µs$^{-/-}$) were more susceptible than the wild-type but showed reduced mortality compared to C1qa-deficient mice [166]. Mice deficient in C1qa or IgM also had higher levels of bacteria in the lung tissue, and higher levels of bacteria in spleen and blood compared to wild-type mice [166]. Mice deficient in MBL of the lectin pathway did not show and increased susceptibility to pneumococcal infections [179]. This is consistent with a report by Brown *et al*. in which mice with impaired classical and lectin pathways due to a deficiency in C4$^{-/-}$ were as susceptible to intranasal challenge as mice deficient in the classical pathway [166]. However, mice deficient in MASP-2, which is required for activation via the lectin pathway, showed reduced survival and higher bacterial counts [179]. This is consistent with the report by Endo *et al.* that ficolin activate the lectin pathway in mice in response to pneumococci [180].

1.7.3.4 The complement system during invasive disease

Mice infected intraperitoneally showed more rapid disease progression if they were deficient in the classical pathway than if they were deficient in the alternative pathway [166]. Mice treated with antibodies inhibiting MASP-2 function showed reduced survival and higher bacterial in load following intraperitoneal infection with pneumococci compared to control animals [179].

During normal conditions there is very little complement proteins present within the central nervous system, however during bacterial meningitis the levels of C3 can increase 20 fold in humans [181]. The importance of the complement system and of the classical activation pathway during infections in the central nervous system has been shown using C1q- and C3-deficient mice. Both mouse strains showed a reduced ability to fight the infection with increased bacterial numbers in the CNS, numbers that were reflected in the secondary bacteremia. Both strains also showed impaired leucocyte recruitment as a consequence. With regard to both bacterial numbers and leucocyte infiltration the C3-deficent strain showed greater impairment [182].

1.7.3.5 Complement deposition in vitro

Incubation of pneumococci with sera from mice with C1qa- and C4-deficiencies led to reduced C3 deposition, whereas factor B-deficiency had little effect on the deposition of C3b. Mice deficient in both the classical and lectin pathway showed deposition similar to that of mice deficient in only the classical pathway [166]. In support of a limited role for the lectin pathway in humans, serum from MBL-deficient human donors showed little reduction in C3b deposition on pneumococci compared from serum from MBL-sufficient donors [179, 183]. The amount of C3 deposition in mice was largely influenced by the alternative pathway whereas the proportion of bacteria positive for C3 deposition was largely determined by the classical pathway [166]. Furthermore, Brown *et al*. observed that the C3 binding to D39 in mouse serum was bimodal with half of the bacteria showing high levels of C3 deposition and the other half with low levels of deposition [166].

The level of C3b deposition inversely correlated to the invasiveness following intraperitoneal injection into mice [184]. Factor H binding has also been shown to be inversely correlated to C3b deposition as well as to the association with human neutrophils. Furthermore, capsular types which bound more factor H were associated with higher attack rates in children [185]. By creating isogenic mutant expressing different capsule alleles, the capsule has been shown to be very important in the resilience of the pneumococcus against C3b deposition [184, 185]. However, the genetic background of the strains expressing the same capsule, show that the genetic contribution outside the capsule locus also plays a role [186]. This shows the complex interplay between bacterial factors which contributes to the resilience of the pneumococcus to complement deposition.

The multifactorial nature of both complement activation and evasion of complement deposition by the bacterium makes it difficult to elucidate the relative contribution of bacterial factors in the context of different complement pathways. Therefore our understanding of the role of complement pathways in the context of pneumococcal infections is far from complete.

1.7.4 Recognition receptors

The key to unlock the ability of the immune response to fight invading microbes is the ability to recognize and respond to potential pathogens. The innate immune system relies on the recognition of pathogen-associated molecular patterns (PAMPs). The PAMPs are recognized by a series of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) (Figure 4). These PRRs are expressed by a wide range of cells including immune cells.

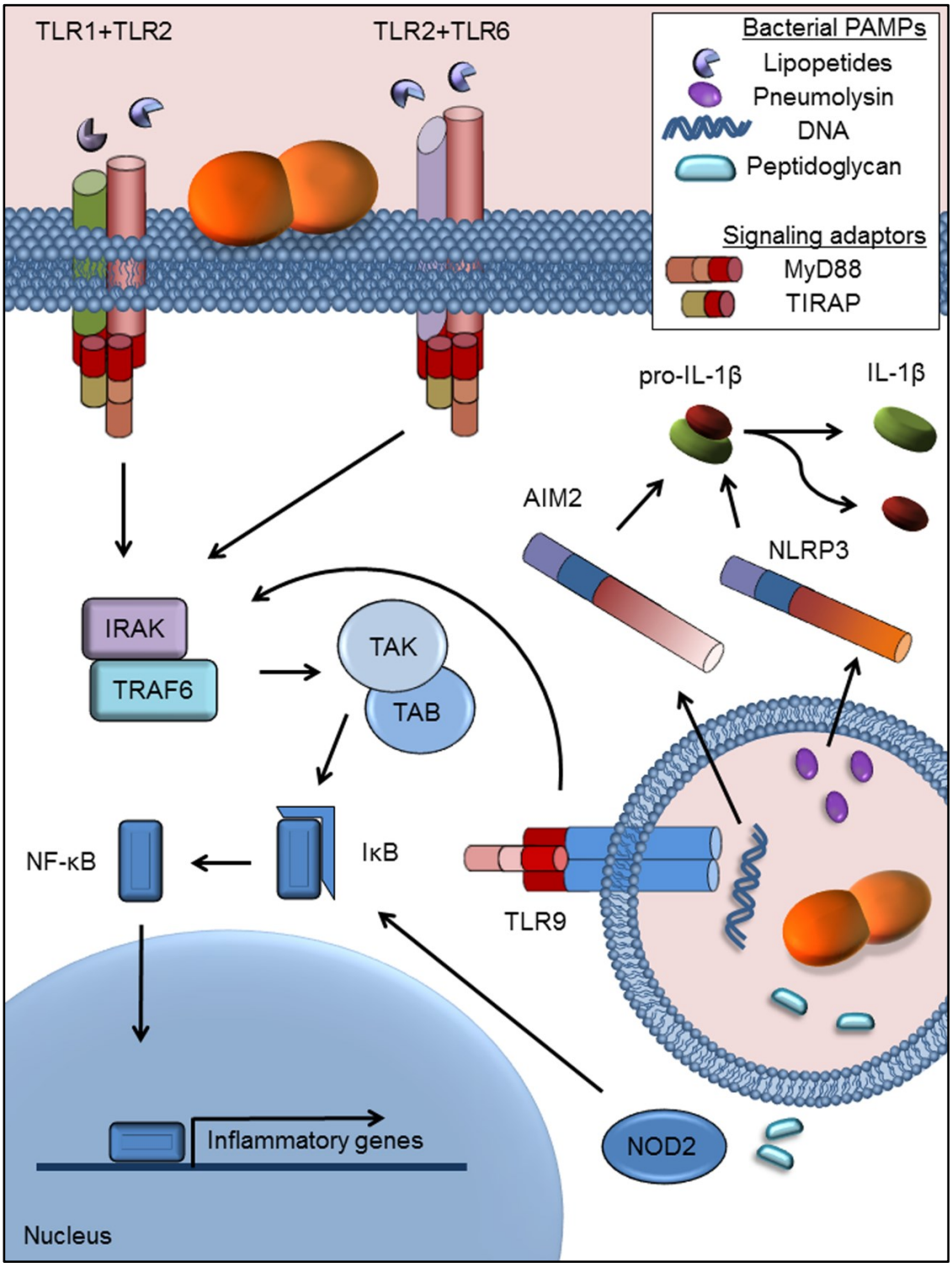

Figure 4. Overview of the PRRs involved in the recognition of *S. pneumoniae* and their signaling pathways. Heterodimers of TLR1 and TLR2 or TLR2 and TLR4 recognize tri- and di-acylated lipopeptides, TLR9 recognizes hypomethylated DNA. Binding of bacterial receptor ligands by TLR leads to the recruitment of adaptor proteins MyD88 and TIRAP, which associate with the TIR domain (red). The endosomal TLR9 receptor does not require TIRAP. The signaling cascade leads to the translocation of the NF-κB to the nucleus and transcription of pro-inflammatory genes. The cytosolic receptor NOD2 recognizes bacterial peptidoglycan fragments in the cytosol and activates NF-κB. The AIM2 and NLRP3 inflammasomes recognize bacterial DNA and pneumolysin respectively. Upon binding of the ligand the inflammasomes cleave pro-IL-1β into active IL-1β.

1.7.4.1 Induction of cytokine production

The TLRs share homology with the IL-1R receptor in the intracellular signaling part of the protein, the Toll/IL-1R domain (TIR). When the pneumococcus stimulates TLRs the cytoplasmic TIR domain of the receptor recruits the adaptor molecules myeloid differentiation primary-response protein 88 (MyD88) and TIRAP. The TIR domains of MyD88 interacts with the TIR domain of the TLR, and via its death domain MyD88 recruits the IL-1 receptor-associated kinase 4 (IRAK-4) which mediates the phosphorylation of IRAK-1. For signaling over TLR2 and TLR4 a second adaptor molecule TIRAP is necessary for the initiation of the signaling cascade [187, 188]. IRAK-1 associates with TNF-receptor-associated factor 6 (TRAF6). TRAF6 activates transforming-growth-factor-β-activated kinase 1 and TAK1-bindng protein 1 complex (TAK1/TAB1-3) which increases the activity of the IκB kinase and leads to the degradation of IκB, the inhibitor of the transcription factor NF-κB [187]. This triggers the translocation of NF-κB from the cytosol to the nucleus. The activation results in the localization of the NF-κB transcription factor to the nucleus, by triggering the degradation of IκB. NF-κB induces the transcription of genes with the NF- κB motif, which are involved in the inflammatory response including genes for the production pro-inflammatory cytokines. The NOD receptors interact directly with NF-κB to induce signaling [189].

The AIM2 and NLRP3 inflammasomes are cytosolic protein complexes that recognize bacterial ligands present in the cytosol. In response to their cognate ligand they cleave the inactive pro-forms of IL-1β and IL-18 into the functional variants. The inflammasomes require the adaptor protein ASC and caspase 1, which mediates proteolytic cleavage of the immature pro-forms of the interleukines. [190].

The most prominent pro-inflammatory cytokines produced in response to a pneumococcal infection are TNFα, IL-1β and IL-6 produced by lung epithelial cells and macrophages [191-193]. The main producer of TNFα in the lung is alveolar macrophages [191, 192]. These cytokines stimulates the acute phase response and activate the production of acute phase proteins in the liver, including CRP. They also activate macrophages and are important for the activation and recruitment of neutrophils.

1.7.4.2 Toll-like receptors and the Inflammasomes

TLRs are expressed by many cell types including epithelial cells, monocytes, macrophages and B lymphocytes, but the type of TLR expressed varies between cell types. TLR2 can form heterodimers with TLR1 and TLR6 that recognize tri-acylated and di-acylated lipoproteins respectively [194]. Alveolar macrophages deficient in TLR2 showed reduced secretion of the pro-inflammatory cytokines IL-1β, IL-6, TNFα and KC in response to *S. pneumoniae* consistent with an observed reduction in inflammation [191]. However, no significant contribution of TLR2 in the clearance of bacteria during pneumonia could be seen [191]. In a

separate study TLR2$^{-/-}$-deficient mice show reduced ability to prevent long-term colonizing pneumococci from causing pneumonia, with significant differences in bacterial numbers three weeks after infection [195]. In line with this study is the observation TLR2-deficinet mice infected with wild-type pneumococci did not show increased susceptibility compared to wild-type mice. In contrast, TLR2-deficient mice infected with a pneumolysin-deficient strain, were unable to contain bacterial numbers compared to wild-type mice [196]. This finding suggests an important role for pneumolysin in triggering the host response.

TLR4 has been suggested to act as a receptor for pneumolysin, and the recognition of pneumolysin by TLR4 has been suggested to induce apoptosis [197, 198]. However, more recently it has been shown that oxidative stress triggered by pathogens generated oxidized phospholipids triggered a TLR4-dependent host response leading to inflammation and apoptosis [199]. The role of TLR4-signaling in response to pneumolysin is increasingly debated, as a number of publications have shown that the NLRP3-inflammasome is responsible for the recognition of pneumolysin [200-202]. The activation of the NLRP3-inflammasome is dependent on the cytolytic activity of pneumolysin [202-204]. An additional inflammasome the AIM2 has been implicated in recognition of cytosolic DNA from *S. pneumoniae* [205].

TLR9 is localized to endosomes and sense hypomethylated CpG motifs present on bacterial DNA. The importance of TLR9 in pneumococcal pneumonia has been demonstrated, as murine alveolar macrophages deficient in TLR9 show reduced uptake and killing of bacteria [206]. This corresponded to increased susceptibility to pneumococcal pneumonia and more disseminated disease in mice [206]. Morphine induced impairment of TLR9 signaling has been shown to increase the mortality following pneumonia and lead to more disseminated disease. This correlated with a reduced production of the chemokine MIP-2 by alveolar macrophages and hence reduced infiltration of neutrophils [207].

In spleen TLR2, TLR4 and TLR9 contributed little in isolation to the response against pneumococci, yet loss of multiple pathways abrogated the response [208]. Another argument for the importance of TLRs, yet redundancy for any single TLR, is the sensitivity seen when the MyD88 adaptor protein is deleted. MyD88-deficient mice show poor ability to control pulmonary pneumococcal infections [209]. MyD88-deficient mice developed both pneumonia and disseminated disease after pulmonary infection with a non-encapsulated strain [210].

1.7.4.3 Nucleotide-binding oligomerization domain 2

NOD2 is an intracellular cytosolic receptor which detects muramyl dipeptide constituent of peptidoglycan and contribute to the pro-inflammatory response to pneumococci [131, 211]. NOD2 plays a role in clearing colonizing pneumococci in mice by stimulating macrophages to secrete the chemo attractant CCL2 and IL-6 [212]. The inflammatory response triggered by

TLR2 and NOD2 sensing of peptidoglycan and its muramyl dipeptide constituent has been shown to cause apoptosis in neuronal cells, and contributes to the neuronal damage seen in mouse models of pneumococcal meningitis [213].

1.8 PNEUMOCOCCAL VIRULENCE FACTORS

1.8.1 The capsule

The composition of the polysaccharide capsule and its importance in pathogenesis has been extensively studied for more than a century. The importance of the capsule transects many different aspects of pneumococcal biology, including epidemiology, medicine and molecular pathogenesis as well as the metabolism of the pneumococcus. It is well known that the capsule is important during systemic disease but it also serve an important function during colonization [214].

Most pneumococcal serotypes carry a negatively charged capsule, which is the major determinant of overall surface charge [215]. It is likely that the negative charge of the capsule helps to prevent binding to the negatively charged sialic acids in mucus by electrostatic repulsion, and thereby reduces mucocillary mediated clearance [216]. A more negative surface charge has also been shown to mediate resistance to killing by human neutrophils [215].

Exceptions to the general rule that the capsule is negatively charged does exist as the capsules of serotypes 7A, 7F, 14, 33F and 33A are not charged [217]. The capsule of serotype 1 differs from that from other capsular types as it incorporates 2-Acetamido-4-amino-2,4,6-trideoxy-D-galactose (AAT-Galp), making the capsule zwitterionic [218].

The capsular locus is located between the *dexB* and *aliA* genes on the chromosome, and ranges in size from 10 kb for serotype 3 to 30 kb for serotype 38 [219]. All but two serotypes, serotype 3 and 37, utilize the wzy-dependent mechanism for synthesis, whereas serotype 3 and 37 utilize the synthase dependent mechanism. The genetic locus and biosynthesis capsule has been reviewed in Yother 2004 and Geno *et al.* 2015 [48, 220]. It is suggested that the capsular locus is transcribed from a single promoter, and regulated by the first gene in the operon *cpsA* [221]. The genes *cpsB*, *cpsC*, *cpsD* are downstream of *cpsA,* and followed by *cpsE* in certain serotypes. These genes are followed by a varied set of genes required for sugar biosynthesis, polymerization, transport as well as modifying enzymes [219].

The wzy-dependent mechanism of capsule synthesis involves the sequential assembly of repeating units sugars, anchored to the inner leaflet of the cell membrane by undecaprenyl phosphate. The synthesis is initiated by CpsE in the majority of serotypes, or by other glycosyltransferases in the other serotypes, and is phosphoregulated by CpsB, CpsC and CpsD. The complete repeat unit is then flipped to the outside of the cell membrane by the

Wzx encoded transporter. On the outside of the cell the growing chain is added to the new repeat unit by the Wzy polymerase [48, 220]. When synthesized by the wzy-dependent pathway the capsule remains covalently like cell wall [222].

The synthase-dependent synthesis occurs in serotype 3 and 37 [223]. This mechanism generates simple polysaccharides which for serotype 3 consisting of glucose and glucoronic acid, unlike the 18 different sugars which can be used in wzy-dependent synthesis [223]. In this process one gene, w*chE,* is reponsible to initiate, polymerize and translocate the polymer. The synthesis of the polysaccharide chain is made by the continuous addition of single molecules of glucose and glucoronic acid. The polymer is concomitantly transported across, but remains anchored to the membrane, although [220].

1.8.1.1 Phase variation and modulation of capsule expression

The production of capsule has been shown to be decreased in response to oxygen and in contact with host cells [224, 225]. The amount of capsule produced is also dictated by the phenomenon of stochastic phase variation by which *S. pneumoniae* displays two phenotypic variants, transparent and opaque. Much about the nature of the phase variation is unclear, but it is suggested to be governed by a type I restriction-modification system leading to alterations in methylation patterns and thereby gene expression [226]. The opaque variant is selected for during invasive disease in mice and expresses a higher amount of capsule. Depending on the serotype the amount of capsule can be in excess of 5 times higher than for the transparent variant[227]. The opaque variant also expresses more PspA [228], and has reduced production of hydrogen peroxide [229]. The production of capsule may be linked to production of hydrogen peroxide as inhibition of the gene for pyruvate oxidase, *spxB*, has been reported to increase the capsule production [230]. The transparent variant produces lower amounts of capsule and up to 4 times more teichoic acid [227]. As transparent variants also expresses more PspC and adheres better to host cells [225, 228], the phenotype is likely an adaptation for colonization of the nasopharynx [231].

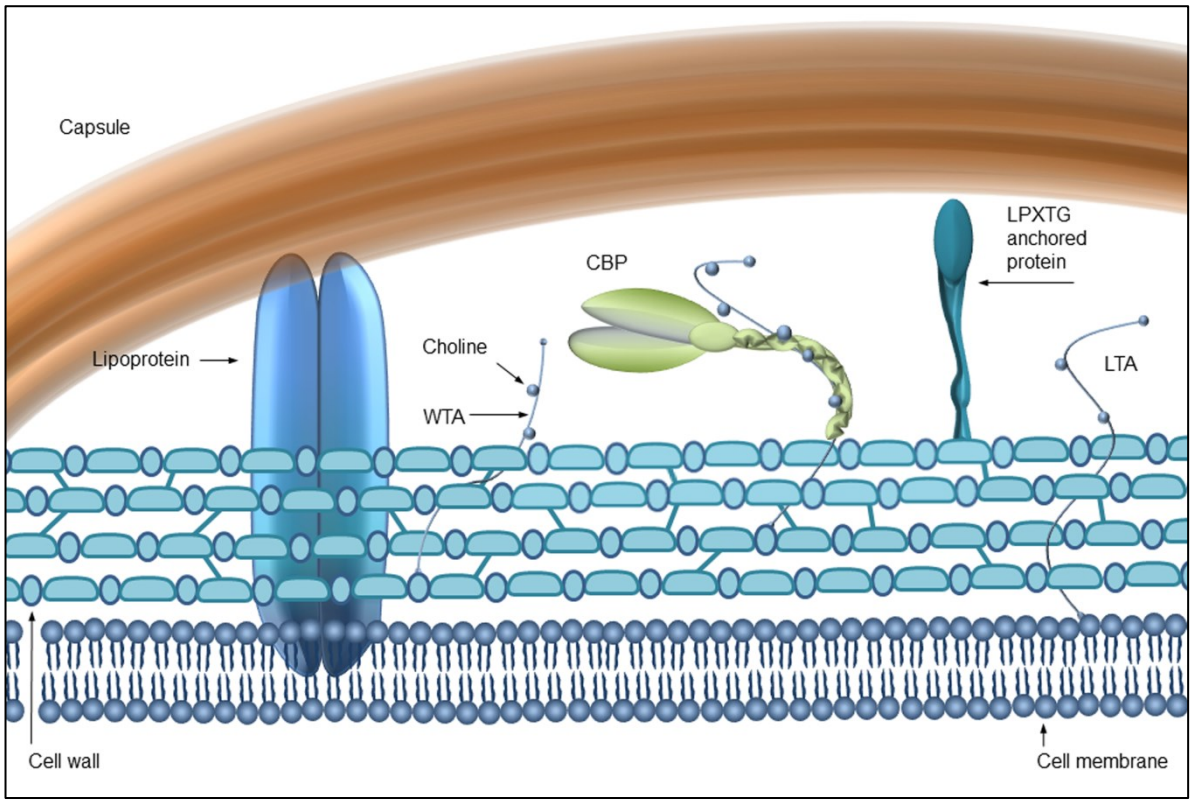

Figure 5. Schematic illustration of the cell surface of the pneumococcus. Lipoproteins are associated to the cell membrane whereas proteins containing a LPXTG-motif are bound to the cell wall. Wall teichoic acids (WTA) attached to the peptidoglycan cell wall and lipid teichoic acids (LTA) anchored to the cell membrane contain choline residues, which serve as attachment points for choline binding proteins (CBPs). The capsule extends from the cell wall (the outer boundary is illustrated in the figure).

1.8.2 The cell wall

As other Gram-positive bacteria *S. pneumoniae* is surrounded by a thick cell wall comprised of a peptidoglycan layer, approximately 16-25 nm thick [232], made up of glycan strands and pepetides. The glycan strands consists of a repeating disaccharide unit of *N*-acetylmuramic acid and *N*-acetylglucosamine (MurNAc-GlcNAc), joined via a β1-4 linkage [233]. Glycan strands are given additional rigidity by cross-links of bridging peptides [234]. The cell wall also contains wall-teichoic acids which are covalently bound to the peptidoglycan, and lipoteichoic acids which are anchored to the lipids of the plasma membrane. The lipoteichoic and the wall-teichoic acids are structurally identical in *S. pneumoniae* and consist of a repeated motif of a phosphate and a pentasaccharide unit. The teichoic acids also contain phosphorylcholine which serves as attachment points for choline binding proteins (Figure 5) [235].

1.8.2.1 Receptor for platelet-activating factor binds to choline

The platelet-activating factor receptor (PAFr) is expressed on epithelial cells in the nasopharynx and lung as well as on endothelial cells. PAFr binds to phosphorylcholine present on pneumococci [236]. PAFr has been suggested to mediate translocation of pneumococci between physiological compartments as it binds phosphorylcholine present on

the teichoic acids of pneumococci [237]. The binding to the PAFr by *S. pneumoniae* is inhibited by physiological concentrations of human CRP which also binds to phosphorylcholine on the bacterium, but CRP binding is in turn inhibited by surfactant [238]. It has been shown that uptake of cell-wall fragments by the PAFr on cardiomyocytes impairs the contractility of these cells during invasive infection [239]. Binding to the PAFr is not only suggested to mediate translocation in the lung but also to be a main route in pneumococcal meningitis by mediating translocation across the blood brain barrier [240]. The PAFr has also been shown to mediate uptake of pneumococcal cell wall fragments [239]. However, it has also been suggested that pneumococci that adhere to endothelial cells co-localize with pIgR but not with PAFr [241].

1.9 SURFACE EXPRESSED VIRULENCE FACTORS

The term virulence factor is given to a bacterial molecule or strategy which, when it exerts its function during the infection process, contributes to the ability to cause disease. It thus can include factors which have no direct role in the interaction with the host immune system, but allows for the bacterium to adapt to the environment within the host. Based on STM screens approximately 300 pneumococcal genes have thus far been implicated to have a role in the virulence of the pneumococcus, [9, 242].

1.9.1 Modes of decorating the bacterial surface

1.9.1.1 Lipoproteins

Lipoproteins are covalently linked to the phospholipids of the cell membrane. The attachment is catalyzed by the signal peptidase II on proteins expressing am N-LXXC-motif, which allows for the protein to be anchored to the surface. There are around approximately 40 predicted lipoporteins in *S. pneumoniae* [243, 244].

1.9.1.2 LPXTG-linked proteins

There are 13 proteins with a LPXTG motif in R6 and 19 in TIGR4 [244]. The proteins of the LPXTG-family have a C-terminal sorting motif which is recognized by sortases and directs the covalent linkage to peptidoglycan. Sortase A is the house-keeping sortase, whereas the sortases B, C and D are involved in pilus formation [245]. Consequently, deletion of the gene encoding sortase A reduces virulence *in vivo* models of colonization, pneumonia and bacteremia [246]. Sortase A has therefore been suggested as a vaccine candidate [247].

1.9.1.3 Choline-binding proteins

The most extensively studied class of surface attached proteins is the choline-binding proteins. There are 12 proposed choline-binding proteins in R6 and 14 in TIGR4 [244]. The choline binding domain consists of around 20 amino acid-long choline-binding sequence

present in tandem up to a dozen times [244]. This motif is conserved in the class with the exceptions of LytA, LytB and LytC. LytA lacks a choline-binding domain, and in LytB and LytC the domain is located at the C-terminus instead of the N-terminal as in other choline-binding proteins. In the case of PspA and PspC the choline-binding domain is preceded by a proline-rich linker sequence [248, 249]. The C-terminal ends of most choline-binding proteins containing a signal peptide that allows them to be translocated across the cell membrane by the Sec translocase [250, 251].

1.9.2 Virulence factors

1.9.2.1 Pneumococcal surface protein A

PspA is a choline-binding protein, which displays high sequence variability. Based on the sequence of its α-helical and charged domain, present in the core region of the protein, the allele can be divided into different families and subdivided into clades. The great difference in the gene suggests that gene families may have different origins. Each family can be subdivided into monophyletic clades based on the amino acid sequence of the B-region [252]. This classification is used in paper I.

PspA is involved in the protection against the complement system by interfering with deposition of C3b. The effect of PspA was reduced in mice lacking factor B implicating the alternative pathway [253, 254]. One way PspA inhibits the classical pathway of complement activation is by binding to phosphorylcholne on the bacterial surface, thereby competing with C-reactive protein for binding [255]. PspA also interferes with the classical pathway, by reducing C1q binding, and with the alternative pathway through an unknown mechanism [256, 257]. By binding human lactoferrin PspA may allow the pneumococcus to scavenge iron on mucosal surfaces, where the availability of free iron is limited [258-260]. It has also been shown that PspA can bind to apolactoferrin, the non-ferrous form of lactoferrin, and in doing so protects the bacterium from the bactericidal effect of the molecule [261]. PspA is highly immunogenic in humans [89, 262, 263], and is ubiquitously found in pneumococcal isolates [252, 264]. Furthermore, immunization with purified PspA has been shown to elicit protection against intraperitoneal challenge in mice [265]. For these reasons PspA is suggested as a vaccine candidate [266]. However, the high sequence variability of PspA also makes it antigenically diverse [252].

1.9.2.2 Pneumococcal surface protein C

Pneumococcal surface protein C (PspC) is also known as choline-binding protein A (CbpA), factor H inhibitor of complement (Hic), C3-binding protein A (PbcA) and *Streptococcus pneumoniae* secretory IgA-binding protein (SpsA). Most pneumococcal strains encode a single copy of the *pspC* gene, but two gene copies can also be present. Based on sequence homology *pspC* has been subdivided into 11 groups. The proteins are attached to the bacterial surface either via a LPXTG-motif or a choline binding domain [267]. Depending on the

family the length of the gene varies greatly from approximately 2-10 kb. The protein is predicted to consist largely of α-helices, apart from the anchoring domain [267, 268].

PspC is nearly as varied in name as it is in ascribed function. It plays an important role in adherence of pneumococci to the nasopharynx of rodents [228, 269], in the establishment of pneumonia [269, 270], and during bacteremia [271]. In adhesion experiments PspC has been shown to mediate adherence to human nasopharyngeal and lung epithelial cells [228, 268]. PspC is able to mediate adherence directly to host tissues, by binding vitronectin and the laminin receptor [272, 273]. However, the adherence to host tissue is increased in the presence of human secretory IgA and factor H [274]. PspC can also bind to the ectodomain, known as the secretory component, of the human poly-IgA receptor (pIgR), but not to the secretory domain of mice IgA [275, 276]. The binding of pneumococci to pIgR results in invasion into human epithelial cells [275, 277]. The invasion into epithelial cells has been suggested to offer a way of translocating across the epithelial barrier [277].

PspC is also able to bind human factor H, but not murine factor H [278]. The binding does not compete with binding to the secretory component of pIgR [279]. Binding of factor H correlates with protection against deposition of complement factor C3, resulting in evasion of activation of the alternative pathway of the complement system and reduced opsonophagocytosis [185, 279-282]. Binding of factor H also increases the ability of the bacterium to adhere to human lung epithelial cells as well as to endothelial cells [283, 284]. In an experimental human pneumococcal carriage model high mucosal levels of factor H correlated with an increased colonization density. A synergistic effect was reported with viral infection and the level of factor H in colonization of the upper respiratory tract. Viral infection alone was associated with a 2.8-fold increase in the odds of becoming colonized, whereas a doubling of the factor H level was associated with a 2.5-fold increase in the carriage density [93]. The corresponding values for the combined association of viral infection and factor H level was 9.3 in the odds of carriage and 4.3 for the carriage densities.

The importance of PspC in the development of colonization, pneumonia and bacteremia is well established. It is a multifunctional protein, able to mediate adhesion and complement evasion. Its selectivity in interacting with human pIgR and Factor H but not their murine counterparts, may offer a partial explanation to species tropism and the resilience of mice to pneumococcal pneumonia. The synergistic association between factor H levels and viral infection may suggest a mechanism by which viral infections leads to a predisposition to subsequent pneumococcal infection.

1.9.2.3 Pneumococcal surface adhesin A

PsaA is a lipoprotein belonging to an ABC transporter complex, which forms a polycistronic transcript *pcaBCA* and together with *psaD* forms an operon [285]. As a transporter it is involved in uptake of Mn^{2+}, and mutants require medium supplemented with Mn^{2+} for

optimal growth [286-288]. PsaA is ubiquitously found in pneumococcal serotypes [289]. The ability of PsaA coated particles to adhere to human nasopharyngeal cells, and a corresponding reduction in the adherence when blocking the PspA interaction, has been demonstrated [290-293]. Mouse and human E-cadherin interacts with PsaA and has been implicated as the ligand for PsaA on human nasopharyngeal cells [291]. The binding is only efficient for the transparent phase-variant [290, 291], which also expresses higher levels of PsaA [294]. PsaA deficient mutants are less virulent in murine models of invasive disease and have a reduced ability to colonize the nasopharynx [286, 295]. Antibodies against PsaA can offer mice partial protection against pneumonia and invasive disease [296].

It is clear that PsaA is an important virulence factor, with a role in both the uptake of Mn^{2+}, an essential micronutrient for optimal growth, and in adherence to cells. It is thus difficult to discern which role attributes to fitness defects of PsaA-lacking mutants to *in vivo*. In one study it was possible to rescue the growth phenotype of PsaA-deficient mutants by administering Mn^{2+} intraperitoneally, but not in a model of otitis media [295]. It has also been reported that the reduced ability to colonize the nasopharynx is due to the lack of a functional manganese transporter, indicated by mutants lacking *psaB* and *psaC* but encoding *psaA* [286, 297]. It is worth to mention that the concentration of Mn^{2+} varies greatly between physiological compartments, with concentrations of around 36 µM found in human saliva [298], but with a concentration of only 21 nM in serum [299]. *In vitro* the addition of 2 µM of $MnSO_4$ was sufficient for optimal growth in a *psaA*-deficient mutant [285]. The high concentration of Mn^{2+} in saliva may suggest that the Mn^{2+} concentration in secretions is not limiting during colonization of the nasopharynx, and that the effect of PsaA during these conditions is due to the role as an adhesin.

1.9.2.4 Lytic amidase A, B and C

S. pneumoniae encodes three autolysins, the hydrolases LytA, LytB and LytC. The major autolysin of the pneumococcus is the highly conserved LytA, which displays optimal activity at 37°C [300]. LytA is responsible for the autolysis seen in late stationary phase (Figure 6), which can be triggered by nutrient starvation or by antibiotics. LytA is a *N*-acetylmuramoyl-L-alanine amidase, largely confined in the cytosol and only present in low amounts on the surface of cells during logarithmic growth. LytA-mediated lysis appears to be controlled by the cell wall synthesis machinery that seems to restrict the access of LytA to its preferred substrate, the nascent peptidoglycan [301]. However, as LytA lacs the signal peptide necessary for secretion across the cell membrane it accumulates in the cytoplasm. At the onset of autolysis large amounts of LytA is thus released into the medium, triggering a fratricidal chain reaction [302].

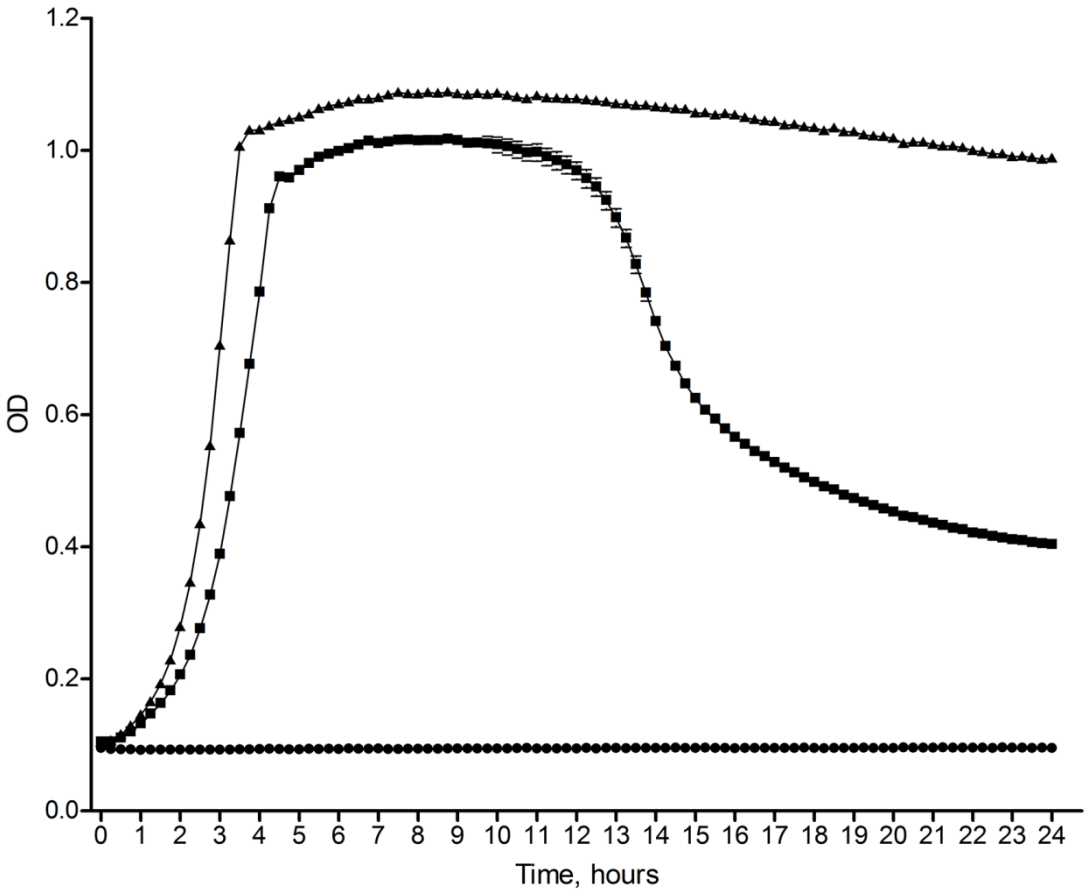

Figure 6. Growth curve illustrating the phenomenon of autolysis in the wild-type strain TIGR4 (■), which is absent in the *lytA* deficient mutant strain TIGR4*ΔlytA* (▲). Culture medium without bacteria was used as control (●). Bacteria were cultured in C+Y medium supplemented with 1% horse serum at 37°C, the optical density (OD) was measured at 600 nm every 15 minutes for 24 hours.

LytA is involved in executing lysis triggered by both penicillin and vancomycin. In the case of β-lactams, such as penicillin, cell wall synthesis is inhibited by binding to the active site of PBPs [303, 304]. For vancomycin the effect is achieved by preventing cross-linking of the peptidoglycan strands, but tolerance to vancomycin do not require alternations in the D-alanine-D-alanine muropeptide target of vancomycin [100]. The mechanism by which the stalling of cell wall synthesis leads to induction of lysis remains unclear.

Mutants deficient in LytA show reduced virulence in mice after intraperitoneal injection and in models of pneumonia [305, 306]. LytA has also been shown to contribute to meningitis in rats [307]. The function by which LytA confers virulence is not known, but several possible explanations have been suggested. By killing neighbouring pneumococcal cells it is possible for naturally competent pneumococci to scavenge potentially useful DNA, not only from other pneumococci but also from related species such as *S. mitis* and *S. oralis* [308]. Lyis has also been suggested to lead to the release of cell wall fragments and microbial proteins involved in immune evasion [309]. Another possibility is that LytA mediates release of virulence factors such as pneumolysin, but autolysis is not an absolute requirement for release of pneumolysin [310-312].

LytB is a glycosaminidase responsible for separation of the daughter cells during cell division, and inhibition of LytB function results in the formation of bacterial chains of excessive length [300, 313]. Increased chain length results in higher complement deposition and in increased susceptibility to opsonophagocytosis by human neutrophils [314]. Longer chains also have an increased ability to adhere to human lung epithelial cells and to colonize mice [315]. The LytB enzyme shows highest activity at 37°C, whereas 30°C is optimal for LytC activity [300]. Similarly to what is seen for LytA, an effect of exogenously added LytC is only seen in stationary phase [313]. LytC also contributes to fratricide and to the release of pneumolysin *in vitro* [311, 316]. The lower temperature optimum for LytC may indicate that the function of the enzyme is primarily localized to the nasopharynx where the temperature is lower. Both LytB and LytC deficient mutants have reduced ability to colonize infant rats, at 30°C LytC mutants also displayed reduced adherence to human nasopharyngeal cells [317].

1.9.2.5 Exoglycosidases – NanA, NanB, BgaA and StrH

Neuraminidases, such as NanA, cleave sialic acids. NanA was found to be present in all 342 strains collected from nasopharyngeal carriage, middle ear, blood and cerebrospinal fluid of children by Pettigrew *et al.*, and the percentages for NanB and NanC were 96% and 51% respectively [318]. The role of NanC in virulence has not been characterized. In contrast NanA has been extensively studied, and has been found to be upregulated in the transparent phase-variant, and has been suggested to impair the function of host immune proteins such as secretory component, lactoferrin and IgA2 by desialylation [319]. NanA is also able to desialylate LPS of competing bacterial species such as *Neisseria meningitidis* and *Haemophilus influenzae* [320]. One additional function suggested for NanA is to expose cryptic receptors that facilitate adhesion.

In support of the proposed function to facilitate adhesion, the absence of NanA reduces adherence to human nasopharyngeal and lung epithelium [321]. This is supported by the observation that NanA-deficient mutants have a reduced ability to colonize the nasopharynx of mice [322]. NanA is not the only glycosidase of importance encoded by pneumococci, as both NanB as well as NanA are important for sustaining bacterial levels in nasopharynx, trachea and lungs of mice, with a greater effect attributed to NanA [323]. During systemic disease the roles reported for NanA and NanB conflict between studies, however a contribution to virulence has been shown. NanA has been shown to have a role in adherence to and invasion of human brain microvascular endothelial cells [324, 325]. Unlike NanA, NanB does not have an LPXTG-motif and has a much lower pH optimum 4.5 compared to 6.5-7 for NanA [326]. It was therefore suggested that the two enzymes perform their respective role in different biological niches. More recent publications suggest that the two enzymes have different substrate specificities, and that NanB is a trans-salidase with specificity for α2-3-linked sialic acids [327, 328]. NanB can thus catalyse the cleavage of Neu5AC, the product of NanA hydrolase, which cleaves α2-3- and α2-6-linked terminal sialic acids at a higher efficiency [327, 328]. Studies have detected sialic acids attached to galactose

via a α2-6-linkage in the nasal mucosa. The α2-3-linkage is found on bronchiolar and alveolar cells were it predominates [329], suggesting a tissue tropism for the two enzymes. The function of NanB in relation to pathogenesis is still to be determined, but its role in conferring the ability of four exoglycosidaes; NanA, NanB, BgaA (β-galactosidase) and StrH (β-*N*-acetylglucoseaminidase) to enable pneumococci to grow on mucin has been demonstrated [330]. This finding points to the importance of these enzymes in enabling the release and subsequent utilization of host monosaccharides as a carbon source.

It has also been shown that NanA can act in a sequential fashion together with BgaA and StrH to cleave human glycoconjugates and thereby facilitate adhesion [331]. NanA and BgaA deficient mutants had a reduced the adherence to human type II lung epithelial cells and to nasopharyngeal cells [331]. NanA, BgaA and StrH deficient mutants were also more susceptible to opsonophagocytosis by human neutrophils [332].

1.9.2.6 PavA, Hyaluronate lyase and α-enolase interacts with the extracellular matrix

The pneumococcal adhesion and virulence factor A (PavA) binds to immobilized but not fluid phase fibronectin, and deletion of *pavA* reduces virulence in systemic infection [333]. Deletion of *pavA* reduced *in vitro* adherence to, and to a corresponding extent invasion of, human type II lung epithelial cells, larynx epithelial cells and endothelial cells [334]. The ability of DCs to phagocytose pneumococci was shown to be reduced by the expression of PavA, and also the release of pro-inflammatory cytokines was reduced [150]. Following intranasal infection PavA deficient mutants were less virulent and were unable to disseminate into blood and cause bacteremia [335]. This corresponded to a reduced ability of the PavA mutant to colonize the nasopharynx immediately after infection and to establish long term colonization. In addition reduced numbers of bacteria were found in the lungs 48 hours after infection compared to the wild-type [335].

Hyaloronic acid is present on human epithelial cells lining the airways, and serve as a carbon source sufficient to sustain growth of *S. pneumoniae* [336, 337]. Hyaluronate lyase breaks down hyaluronic acid to disaccharide units consisting of acetylglucosamine and glucuronic acid, which permits growth at half the efficiency of glucose [336]. Hyaluronic acid is a constituent of the extracellular matrix and is important for maintaining the barrier function of the matrix in the lungs. Hyaluronate lyase has been shown to increase the ability of pneumococci to colonize mice [336].

The α-enolase binds plasminogen and increases the adherence to epithelial and endothelial cells [338]. Plasminogen can be converted to its proteolytic form plasmin, which when it is present on the bacterial surface facilitates the degradation of VE-cadherin causing breakdown of tight junctions between epithelial cells [339].

1.9.2.7 Two types of pili

The type 1 pilus is covalently anchored by an LPXTG-motif and is encoded by the 12 kb *rlrA* pathogenicity islet [340]. The pilus is form a thin flexible structure of approximately 10 nm diameter and 1 μm in length, but can exceed 1.5 μm in length [245, 341]. The operon is found in 30% of pneumococcal isolates, and the pilus is associated with certain clonal complexes [342].

A highly successful clone of ST156, which expanded in Sweden between 2000 and 2003, carried the pilus and was also non-susceptible to penicillin [343].The expansion was mainly caused by an increase in a lineage of serotype 14 which was a capsule-switch of a piliated and penicillin nonsusceptible 9V lineage [343]. The pilus was first identified in an STM screen as mutants were impaired in their ability to colonize and cause pneumonia [344]. Subsequently a reduced ability of pilus negative bacteria to disseminate from lungs in mice was reported [345]. The pilus is an important mediator of adherence to human type II lung epithelial cells, and it increases the proinflammatory cytokines, TNFα and IL-6, after intraperitoneal infection in mice [340].

The pilus operon consists of seven genes; the positive regulator *rlrA* of the islet, the three genes encoding three pilins with LPXTG-motifs; *rrgA*, *rrgB* and *rrgC*, and three genes encoding sortases; *srtB*, *srtC* and *srtD* [340, 346].

The pilus operon is under the negative control of the MgrA transcriptional repressor [347], either by acting on the different genes within the operon or on the rlrA regulator. RlrA is a positive regulator required for transcription from the *srtBCD*, *rrgA* and *rrgBC* promoters, it also promotes transcription from its own promoter [348]. A negative feeback loop is generated by RrgA, which is able to negatively regulate the expression of *rlrA* [349]. The expression of *rlrA* is downregulated in response to Mn^{2+} [350]. Several other regulators have also been implicated in repression of pilus expression such as the growth-phase dependent two-component system TCS09, the manganese-responsive regulator PsaR, the MerR regulator responsive to zinc and other metals and TCS08 [345, 351].

The sortases are involved in the cell wall attachment and assembly of the proteins into a pilus structure [340, 346], although only *srtB* is indispensable for the formation of wild-type pili as it is necessary for the incorporation of RrgC [245]. The major pilus protein is RrgB which forms the stalk of the pilus, RrgA is found clustered along the pilus [245, 341]. RrgA binds laminin, collagen and fibronectin and mediates the pilus-mediated adherence observed to human type II lung epithelial cells [341, 352]. RrgC is found at the tip of the pilus and along the structure as monomer or clusters [245, 346].

In 2008 a second type of pilus was described in pneumococci [353], the function of which still largely remains to be characterized. The pilus is found in 21% of clinical isolates [354].

The region encoding the type 2 pilus is 6.5 kb in length and consists of five genes, two sortases *srtG1* and *srtG2*, *sipA* as well as two genes, *pitB* and *pitA,* containing LPXTG-motifs. The *sipA* gene is believed to encode a signal peptidase [353]. SrtG1 is involved in the polymerization of the PitB subunit which forms the backbone of the pilus. SrtG2 and PitA are not required for the formation of the pilus. The pilus mediates adherence to human type II lung epithelial cells *in vitro* [353].

1.9.3 Distally acting virulence factors

1.9.3.1 Pneumolysin

Pneumolysin is a cholesterol-dependent, pore-forming cytotoxin, which is present in virtually all clinical isolates [355]. Pneumolysin does not have an N-terminal signal sequence commonly associated with cholesterol-dependent cytolysins and required for Sec-dependent secretion. The view was therefore that pneumolysin was released by autolysis. However, there are reports that pneumolysin is released independently of autolysis [312, 356], suggesting active secretion. This secreted pneumolysin is associated with the peptidoglycan cell wall [357]. Thus there seems to be a mechanism of secretion of pneumolysin in addition to the release of cytoplasmic and cell wall associated pneumolysin as a result of cell lysis.

There are 15 described alleles for the pneumolysin locus [358]. An aberrant variant of pneumolysin is mutated in a domain required for pore formation and therefore lacks the cytolytic capability. This variant is found by certain lineages of serotype 1 strains of CC306, in serotype 8 and in non-typeable strains [358, 359].

In primary human epithelial cells isolated from the nasopharynx, incubation with pneumolysin leads to blebbing of cellular membranes and swelling of the cytoplasm. In addition, pneumolysin caused a reduction in the frequency of cilia beating, and disrupted tight junctions in between cells [360]. The effect on the frequency of cilia beating and disruption of the integrity of the epithelium was dependent on the cytolytic activity of pneumolysin [361]. In mouse models of pneumonia pneumolysin has been described to have a strong effect during the early stages of infection during which it contributes to bacterial outgrowth, and acts as an important factor for the pathology associated with pneumococcal pneumonia [362-364]. Pneumolysin also contributes to the dissemination of pneumococcal pneumonia to bacteremia and to the severity of disease during invasive infections [362]. Further stressing the importance of pneumolysin in the establishment of infection is the partial protection against subsequent pneumococcal infection seen in mice after immunization with pneumolysin [365].

Exogenously added pneumolysin to pneumolysin-deficient strains increased the disruption of the lung epithelium of infected mice and increased bacterial numbers to levels comparable to the wild-type [362]. Similarly, in *ex vivo* experiments recombinant pneumolysin was

sufficient to disrupt the tight junctions that maintain the integrity of the epithelial layer [366]. Pneumolysin is therefore likely to play an important role not only in the pathology of disease, but by disrupting the barrier function of the lung epithelium also in the ability of pneumococci to invade the bloodstream.

Pneumolysin drives inflammation by stimulating the release of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNFα and the production of nitric oxide in macrophages [200, 367, 368]. Neutrophil transmigration across a human endothelial cell layer is also induced by pneumolysin.

Pneumolysin has been shown to induce the classical and lectin pathways of complement activation. As pneumolysin is released from the bacterium it can freely diffuse and complement activation is therefore likely to occur distally from the bacterium. Activation of the classical pathway of the complement system in human and mouse can be induced by pneumolysin in the presence of non-specific IgM and IgG3 antibodies, and without the requirement of pneumolysin-specific antibodies [369]. Pneumolysin is also able to activate the lectin pathway by binding to human L-ficolin [369]. In rats a pneumolysin expressing strain was shown to reduce the levels of serum complement factor C3 compared to a pneumolysin-deficient mutant. The sera of cirrhotic rats were also shown to be less able than wild-type serum to opsonize bacteria for phagocytosis by neutrophils [370]. Likewise pneumolysin reduces the level of C3 deposition by the classical pathway on bacteria incubated with human serum [256]. It has been reported that patients with pneumococcal disease have reduced levels of complement proteins [371].

1.9.3.2 Hydrogen peroxide

S. pneumoniae is able to produce high amounts of hydrogen peroxide (H_2O_2). The production of hydrogen peroxide offers a competitive advantage against hydrogen peroxide-sensitive bacterial species, such as *Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus* and *Neisseria meningitidis* during colonization of the nasopharynx [372-374].

Hydrogen peroxide has been shown to inhibit cilia beating, an important defense mechanism, on human primary nasopharyngeal cells and to cause damage to the epithelium [361]. Hydrogen peroxide also inhibits cilia beating on ependymal cells found in the ventricles of the brain, a sign of toxicity [375]. It was recently reported that hydrogen peroxide produced by pneumococci can induce DNA damage and apoptosis in human type II lung epithelial cells [376]. Although the number of bacteria used in the study (MOI ranging from 40-400) is unlikely to accurately reflect the clinical situation.

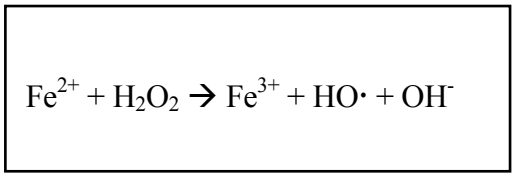

Figure 7. The Fenton's reaction in which iron catalyze the generation of reactive oxygen species.

Under aerobic growth conditions the pyruvate oxidase, encoded by the *spxB* gene, generates high amounts of hydrogen peroxide, which can exceed 1 mM *in vitro*. However, hydrogen peroxide is highly reactive and can lead to the formation of reactive oxygen species (ROS) via Fenton's reaction (Figure 7). Although hydrogen peroxide can lead to the peroxidation of cellular membranes and carbonylation and oxidation of proteins the most reactive species is the hydroxyl radical (HO·), which can directly damage the DNA [377]. The ability of the bacterium to produce hydrogen peroxide thus comes at a risk, and the mutation frequency of pneumococci in the presence of endogenously produced hydrogen peroxide is increased [378].

S. pneumoniae must protect itself from the harmful effects of the endogenously produced hydrogen peroxide. However, pneumococci do not encode the enzymes catalase or NADH-peroxidase which are often used by bacteria to inactivate ROS [6]. Pneumococci also lack regulons, such as OxyR, PerR, RpoS, SoxRS and Mar, which have been implicated in the protective response against oxidative stress in other bacteria [379]. The pneumococcus therefore has to rely on other mechanisms, which remain poorly understood, to tolerate the effects of hydrogen peroxide. It has been shown that the production of hydrogen peroxide is intrinsically linked with the resistance to hydrogen peroxide. Mutants with impaired *spxB* function are more sensitive to hydrogen peroxide, even if pretreated with low levels of hydrogen peroxide to induce potential detoxification pathways [380]. One way of protecting itself from the effects of misfolded and damaged proteins is through degradation of aberrant proteins by proteases, such as HtrA and ClpP. These proteases have been shown to contribute to the tolerance of exogenously added hydrogen peroxide [381, 382]. In addition lipoprotein TlpA has been suggested to function as an antioxidant, repairing membrane proteins and mutants show a ten-fold reduction in viability to exogenously added hydrogen peroxide [383].

The *spxB* gene is involved in the generation of acetyl-phosphate, and the wild-type contained ten times more acetyl-phosphate compared to *spxB* deficient mutants. Killing under extremely high hydrogen peroxide concentrations (20 mM) occurs only after ATP has been depleted [380], indicating a link to energy starvation.

The tolerance of pneumococci to the Fenton's reaction is suggested to be partly mediated by sequestration of Fe^{2+} away from its DNA [380]. Also Mn^{2+} has been shown to play an

important role in protection against ROS. The manganese superoxide dismutase (MnSOD), encoded by *sodA*, is upregulated during aerobic growth and plays an important role in the protection against oxidative stress [384]. Mn^{2+} can function as an antioxidant and this role combined with the requirement of SodA for manganese may explain the observed importance of the PsaA. It may also hint at a role for the putative thiol peroxidase PsaD belonging to the same operon, in the protection against oxidative stress [297, 385].

1.10 BACTERIOPHAGES

Bacterial viruses, so called bacteriophages, can be lytic or lysogenic. In the lytic lifecycle the bacteriophage infect the host cell and use the bacterial replication machinery to multiply. After replication the bacteriophage then triggers lysis of the host bacterium by expressing a lytic enzyme, resulting in the release of phage particles which can then infect bacteria in the surrounding environment. Temperate bacteriophages, or prophages, become stably integrated in the bacterial genome after infection. Prophages are present in more than half of clinical isolates of pneumococci [386, 387]. The prophage remains incorporated as an additional genetic element and is replicated by the host together with the bacterial DNA as the bacterium divides. Some prophages retain their ability to induce lysis, which can be triggered by environmental stress signals [388]. Other prophages loose this ability over time due to genetic mutations, blurring the border between bacteriophage and bacterial genome.

Temperate bacteriophages are known to influence the virulence in several other bacterial species. By carrying genes encoding toxins they contribute to several well-known toxin mediated diseases (so called toxinosis) caused by bacteria. Examples include diphtheria toxin in *Corynebacterium diphtheriae* causing diphtheria, the leucocidin toxin involved necrotizing pneumonia caused by *Staphylococcus aureus*, and scarlet fever caused by *Streptococcus pyogenes* [100].

It has been suggested that the hydrogen peroxide produced by *S. pneumoniae* is sufficient to trigger the lytic cycle of temperate bacteriophages present in some strains of *Staphylococcus aureus*. The induction of temperate bacteriophages in competing microorganism would thus be a mechanism utilized by the pneumococcus to increase its fitness during nasopharyngeal colonization [389].

Two lytic bacteriophages, able to infect *S. pneumoniae* have been studied in some detail. These are the bacteriophages Dp-1 belonging to the *Siphoviridae* family, and Cp-1 of the *Podoviridae* family. The genomes of both Dp-1 and Cp-1 are double-stranded and linear, with respective sizes of ~57 kb and 20 kb respectively [390].

Exploiting the lytic effect of the lytic enzymes encoded by bacteriophages has been suggested as a possible treatment strategy against pneumococcal infections. Animal experiments in rats and mice have shown a therapeutic effect of Cp-1-encoded lysin Cpl-1 in pneumococcal

pneumonia, bacteraemia and meningitis [391-394]. A synergistic effect of more than 1,000 fold between Cpl-1 and the antibiotic lipopeptide daptomycin compared to the drug or lysin alone was observed *in vitro*. A therapeutically effect could also be demonstrated in an *in vivo* model of bacteremia [395]. A synergistic effect has also been shown *in vitro* for Cpl-1 and penicillin for a strain highly resistant to penicillin [396].

2 AIMS

2.1 GENERAL AIMS

The overarching aim of the papers included in this thesis was to explore the complex interplay between the pneumococcus and its host. For this purpose the virulence of clinical pneumococcal isolates was characterized, with a focus on the early stages of the infection process and the innate immune response.

2.2 SPECIFIC AIMS

2.2.1 Paper I

To investigate the potential of pneumococcal serotypes and genetic lineages to cause invasive pneumococcal disease in children. *In vivo* models and *in vitro* assays were used to further characterize the molecular differences between strains representing lineages with different potential to cause disease.

2.2.2 Paper II

To investigate how a defective *spxB* gene influence the virulence properties of a serotype 1 strain, and the ability of the host immune response to clear the bacteria during invasive disease.

2.2.3 Paper III

To characterize how the immune response affects the disease progression to fulminant pneumonia or disseminated disease. The study made use of two pneumococcal strains which showed different tropisms for lung and blood.

2.2.4 Paper IV

To investigate if virulence properties of a bacterial strain can be conferred by genes integrated into the bacterial genome by a temperate bacteriophage.

3 METHODOLOGICAL CONSIDERATIONS

3.1 EPIDEMIOLOGICAL IDENTIFICATION OF PNEUMOCOCCAL ISOLATES

3.1.1 Determination of species and serotype

The following characteristics were used to identify *S. pneumoniae;* colony morphology with α-hemolysis, solubility in bile or deoxycholate, susceptibility to optochin, positivity for reaction with anti-pneumococcal antibodies (serotyping).

All clinical isolates in paper I and paper II were characterized by serotyping at the Public Health Agency of Sweden (prior to 2014 at the Swedish Institute for Infectious Disease Control). Serotyping was performed using gel diffusion method and 46 serogroup antisera (Statens Serum Institut, Denmark). Isolates assigned serogroups where further characterized using type and factor sera. When required the Quellung reaction (capsular swelling) was used to confirm the gel diffusion results. Strains in paper III were obtained from other laboratories and were typed to confirm the serotype. Following *in vitro* and *in vivo* experiments the identity was confirmed by colony morphology including α-hemolysis, and if required testing of optochin sensitivity.

3.1.2 Epidemiological characterization based on molecular methods

3.1.2.1 Multi-locus sequence typing

Multi-locus sequence typing (MLST) is used in epidemiology to discriminate isolates based on genetic differences in seven house-keeping genes. As the method is based on sequencing it is highly standardized and allows for cross-comparisons between different laboratories and studies.

MLST makes use of sequence variations in the following seven house-keeping genes; shikimate dehydrogenase (*aroE*), D-alanine-D-alanine ligase (*ddl*), glucose-6-phosphate dehydrogenase, (*gdh*), glucose kinase (*gki*), transketolase (*recP),* signal peptidase I (*spi*) and xanthine phosphoribosyltransferase (*xpt*). The primers used for the sequencing are detailed in Table 2, and amplify fragments of 400-500 bp in length [397].

Each of the sequenced loci is assigned a number depending on the number of discrepancies compared to a reference sequence. The combination of the allele numbers of an isolate is obtained from an online database and determines the ST of that isolate [398]. Clonal complexes are defined according to the eBURST algorithm, based on the ST number [399]. Isolates within a clonal complex have at least six allele numbers identical to those of at least one other isolate in the same CC. The ST with the most single locus variants in the clonal

complex, and in case of a tie between two STs the larger number of double locus variants, is by parsimony regarded as the predicted founder of the complex [399].

Table 2. Primers used in MLST [397, 400, 401]

Primer	5' - 3' primer sequence
aroE-up1	AAGCTTGATGGCTATACACG
aroE-dn	ATCCATGCCCACACTGG
gdh-up	ATGGACAAACCAGC(G/A/T/C)AG(C/T)TT
gdh-dn	GCTTGAGGTCCCAT(G/A)CT(G/A/T/C)CC
gki-up	GGCATTGGAATGGGATCACC
gki-dn	TCTCCCGCAGCTGACAC
recP-up1	GTTTTCTAGGTATTGACGCC
recP-dn1	TTGCCTGAAGCTTTTGCTGT
spi-up1	CAGCTTCTGCGCCAAATTGG
spi-dn1	GAGATTGGTGATTCTCTGCC
spi-up2	TTATTCCTCCTGATTCTGTC
spi-dn2	GTGATTGGCCAGAAGCGGAA
spi-up3	AGAGTGGGGATTATTCCTCC
spi-dn3	TTCCGATACGGGTGATTCC
xpt-up	TATAGTATTAGCAAAAGGGATC
xpt-dn	CCAAGAACGGCTGCTTGC
ddl-up	AAATGCCTTACGTTGGTTGC
ddl-dn	CAAGGTCAACCAAACGCTC

3.1.2.2 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) relies on fragmentation of the genome in order to generate a band pattern. The similarity of the band pattern reflects the genetic relatedness of isolates. The method of separation was developed by Schwartz and Cantor in 1984 [402]. The DNA of the isolate is digested with a restriction enzyme. The enzyme used can vary between laboratories, and limits the possibility to compare the results. In paper I the restriction enzyme ApaI with the 5'-GGGCCC-3' restriction site was used. The fragments are then subjected to gel-electrophoresis in an electric field with pulsating currents from different angles, to allow for separation of large fragments [403].

The use of different restriction enzymes and variability between users in interpreting the banding pattern, as well as a lack of a standardized nomenclature for naming clones makes it difficult to compare results between laboratories [404, 405].

3.1.3 Calculation of Odds Ratio

The odds ratio (OR) of IPD can be calculated by comparing the number of isolates collected from patients with invasive disease to that of healthy carriers in the same geographic region during the same time period. OR is the ratio of the probability of occurrence of the event to the probability of the event not occurring (Equation 1). The OR for pneumococcal isolates is

often calculated based on serotypes, MLST and PFGE clones. When applied in paper I ORs higher than 1 indicate a high invasive disease potential, i.e. the isolate belongs to a serotype or clone that is associated with invasive disease. Conversely an OR lower than 1 indicates that the serotype or clone is associated with carriage.

$$OR = \frac{\#\text{ invasive isolates of type A} \quad x \quad \#\text{ carriage isolates of types other than A}}{\#\text{ carriage isolates of type A} \quad x \quad \#\text{ invasive isolates of types other than A}}$$

Equation 1. Formula for calculating odds ratio. #; number of A. A; isolates of a particular serotype, MLST or PFGE clone.

3.1.4 Classification and induction of prophages

In order identify isolates harboring functional prophages mitomycin C was used to induce lysis in exponentially growing cultures in Paper I. Mitomycin C induces phage mediated lysis by cross-linking DNA, thereby inducing the stress response and triggering the phage genes involved in lysis. The assay was adapted from previous publications [386, 406].

In paper I and IV a PCR-based method of typing prophages designed by Romero *et al.* was used [386], using PCR primers specific for phage encoded sequences (Table 3). The method was developed based on the analysis of the genomes of ten pneumococal prophages, and classified the phages into three groups [406]. In paper I this method was used to classify the phages in all serotype 6B isolates for which lysis was induced by mitymocyin C. This enabled us to investigate a potential correlation between phage group and invasive disease potential.

Table 3. Primers used to group prophages adapted from [386].

PCR	Target gene	Primer	5' - 3' sequence	Phage group
1	Integrase 1 gene	OXC141-5	CAGATAGGTCAGAGCGTAGAATTG	1
		P5-R	GATACACTGTTCAAACCCTCAATTC	
2	Holin (hol1)	P9	GATTTTGTGACAGGGACGATTG	1, 2, 3
		P9-R	GCGATGTACCCGAGATAGATTG	
3	Integrase 2 gene	PR5 phi23	GCTAAAAACGAAATATGAGAATGCC	2
		PR2 phi23	GTACGGCATAGGCTCAGAGG	
4	Tape measure protein (TMP) gene	PR6 phi23	GGAAGGAGGAAGTAAATGGCAAC	2
		PR7 phi23	CCAACTGCTCCACCAACACC	
5	orf2	MM1-P6	CCTCACACTCTCCTTGGCC	3
		MM1-P7	CCTTTTACATCCCAGCCCTC	
6	Major tail protein gene	MM1-P3	GCTCAGTTGATTGTCCATTTGC	3
		MM1-P4	CGACAACGATGGTCACTTTTCC	

3.1.5 Cloning

Bacterial mutants in paper II and IV were generated by insertion-deletion mutagenesis. In paper II the capsule locus and *spxB* gene were deleted by PCR ligation mutagenesis. Flanking

regions upstream and downstream of the target sequence were amplified with the proximal primers containing ApaI and BamHI restriction sites. The resistance cassette was amplified using corresponding restriction sites. Fragments were then ligated together and PCR amplified using the proximal primers. For complementation the target sequence was amplified using primers with KpnI and ApaI for ligation into the construct in between the resistance cassette and the downstream flanking region. For the kanamycin cassette the ApaI and KpnI were inserted to the upstream fragment and target sequence. In paper IV mutants were generated using the overlap extension method [407]. Primers with overhangs were used to fuse sequences together by PCR. All published mutants were sequenced to verify the constructs.

The assembled constructs were then used to transform *S. pneumoniae*. As previously described the kanamycin and erythromycin resistance markers used in these constructs have been successfully used for cloning in pneumococci [340]. In order to reduce the risk of polar effects contributing to the phenotype of the deletion mutants, deletions were complemented with the sequence of interest to restore wild-type characteristics.

3.1.6 Adherence and phagocytosis of pneumococci *in vitro*

In vitro assays were used in paper II and III to study adherence, phagocytosis and internalization of pneumococci into mammalian cells. To investigate the interaction with phagocytic immune cells, human monocytic THP-1 cells were used in paper II [408, 409]. The THP-1 cells were induced into adherent macrophage-like cells with phorbol myristate acetate (PMA 100 ng/mL) for 30 hours. In paper II the murine macrophage cell line RAW265.7 was used in adherence and phagocytosis assays [410]. To study the ability of pneumococci to adhere to epithelial cells in the respiratory tract the human Detroit 562 nasopharyngeal epithelial and the human A564 type II lung epithelial cell lines were used [411, 412].

Human or murine cells were seeded as monolayers on a plastic surface to which bacteria were added. To study phagocytosis the mammalian cells were incubated together with bacteria to allow the bacteria to adhere. In paper II and III an incubation time of 1 hour with 20 bacteria per mammalian cell was used for macrophages, and 10 bacteria per epithelial cells for 30 minutes, 1, 2 or 4 hours. In order to remove unbound bacteria the cells were then washed. By lysing the macrophages in 2% saponin and plating serial dilutions of the lysate on blood agar plates, the number of colony forming units (CFUs) could be enumerated. The CFUs from the lysate includes both bacteria bound to the surface of the mammalian cells and the bacteria inside the cells. In order to determine the number or bacteria inside the mammalian cell, the external bacteria were killed with 750 µg/mL of gentamicin for 15 minutes. The CFUs after gentamicin treatment correspond to the internalized or phagocytosed bacteria, which can then be used to adjust the CFUs of the lysate to estimate the adherent fraction.

3.1.7 Complement deposition assay

To measure the deposition of opsonizing C3-fragments bound to the surface of bacteria a FACS based approach was used in paper III. The assay does not allow for discrimination of the contribution of different complement activation pathways to the C3-deposition- Furthermore, the assay does not address if a reduced C3-deposition is caused by an reduced ability of the complement system to deposit C3, or if it is caused by the enhanced ability of the pneumococci to limit deposition by expressing factors such as PspC or PspA. The antibody used to detect C3-fragments recognizes C3, C3b, and iC3b and is cross-reactive for both human and murine C3-fragments [413, 414]. Both C3b and iC3b fragments function as opsonins. As a negative control bacteria were incubated in PBS.

3.1.8 In vivo infection models

Within the field of pneumococcal research several different *in vivo* infection models are established, with the mouse (*Mus musculus*) being the most widely used model organism. Models have been used to study the interaction between *S. pneumoniae* and the host during colonization, otitis media, pneumonia, bacteremia and meningitis.

3.1.8.1 Mouse strains

Several different mouse strains have been used to characterize host-pathogen interactions with pneumococci. Different mouse strains show differences in their response and overall susceptibility to pneumococcal infections. Inbred strains commonly used include C57BL/6, BALB/c and CBA/Ca mice. In studies with the *S. pneumoniae* D39 strain BALB/c mice showed low mortality and 5-15% of the mice succumbed after intranasal infection. BALB/c mice were also resilient to bacterial outgrowth in the lungs and to the development of bacteremia following infection, but transient or persistent low level bacteremia occurred [415, 416]. CBA/Ca mice were on the other hand sensitive to invasive disease [416], and C57BL/6 mice showed an intermediate phenotype [415, 417]. The difference between CBA and BALB/c mice correlated with a higher recruitment of neutrophils into the lungs of BALB/c mice during the first 12 hours after infection [415].

Inbred strains are maintained to minimize the genetic diversity and thereby the variation in response between animals. Outbred strains are instead bred to maximum heterozygosity, to allow for a phenotypic diverse response mimicking natural variation in the response to a challenge of the immune system. In pneumococcal research the most frequently used outbred mouse strain is MF1 [306, 363].

In paper I-IV the *in vivo* infections were carried out with C57BL/6 which is an established mouse model in the laboratory [9, 206, 209, 352, 418]. As mentioned the C57BL/6 mice show intermediate sensitivity to pneumococcal infection, which is advantageous during

screening of clinical isolates as both highly pathogenic and non-pathogenic bacterial strains can be assayed. Another benefit is the wide range of genetically modified animals available in the C57BL/6 genetic background.

3.1.8.2 SIGN-R1 knock-out and transgenic CD11c.DTR mice

To investigate the role of immune cells, specific populations were depleted using a transgenic mouse models to study the role of CD11c-expressing cells and a constitutive knock-out model of the SIGN-R1 receptor.

In paper III the transgenic C57BL/6xCD11c.DTR mouse strain was used to deplete CD11c expressing dendritic cells. This mouse model was originally developed in FVB/N mice by coupling the simian diphtheria toxin receptor to the CD11c promoter and therefore allows for the conditional depletion of CD11c-expressing cells. Following a single injection of diphtheria toxin, depletion of approximately 90% of splenic DCs without affecting the number of splenic macrophages or B lymphocytes was reported [419]. The histology of the spleen remained largely unchanged after depletion of CD11c expressing cells, apart from macrophages which showed altered morphology. This was suggested to be due to the involvement of macrophages in clearing apoptotic CD11c expressing cells [419]. Several studies using this transgenic model in the BALB/c background have reported poor depletion of CD11c-expressing cells in compartments other than spleen, as well as neutrophilia induced by diphtheria toxin in the transgenic model [420-422]. The limited extent of depletion of CD11c-expressing cells reported in these previous publications is in line with the observations described in paper III.

In paper II the C57BL/6 SIGN-R1$^{-/-}$ mouse strain was used to investigate the role of this receptor in the clearance of the serotype 1 bacteremia and implicate marginal zone macrophages in the process. SIGN-R1 was first identified when Park *et al.* searched for a mouse homologue to the human dendritic cell receptor DC-SIGN [423]. The study revealed five homologous receptors in mice, murine DC-SIGN and SIGN-R1-4, with around 70% amino acid identity to human DC-SIGN in the carbohydrate recognition domain. Neither of the SIGN-R receptors was expressed on murine DCs. The expression of SIGN-R1 was found to be localized to splenic marginal zone macrophages and to macrophages in the medullary region of the lymph nodes [134, 424]. SIGN-R1 is also expressed on resident peritoneal macrophages [425]. Lanoue *et al.* reported a normal composition of immune cells, with normal localization of marginal zone macrophages and splenic architecture in C57BL/6 SIGN-R1$^{-/-}$ mice [135]. The commonly used murine macrophage cell lines J774.1 and RAW264.7 do not express SIGN-R1 [134].

3.1.8.3 Depletion of macrophages and neutrophils

Administration of liposome encapsulated clodronate is a routinely used method to deplete macrophage populations. If the substance is administered intranasally or directly into the

trachea it is able to deplete in excess of 80-94% of alveolar macrophages [207, 426, 427]. Depletion of splenic macrophages is highly efficient when lipsome encapsulated clodronate is administered intravenously, but also affects marginal metallophillic macrophages in the white pulp and macrophages present in the red pulp [424, 428].

Depletion of neutrophils by intraperitoneal administration of anti-Ly6G antibody (clone 1A8) is well established and results in a decrease of circulating neutrophils as well as neutrophils infiltrating into the lung after pneumococcal challenge [427, 429]. Most studies use a single injection of antibody. We found that repeated injections prior to infection prevented neutrophil release from the bone marrow following neutrophil challenge. In paper II and III we therefore administered 0.5 mg anti-Ly6G antibody by intraperitoneal injection three times before infection, at 24, 16 and 0 hours. This resulted in a 78-88% depletion of circulating neutrophils.

3.1.8.4 Route of infection

Pneumococcal pneumonia and colonization can be studied using the intranasal route of infection. The usual procedure is to apply a bacterial suspension to the nares of the animal, the suspension is then aspired into the nasal cavity and to a lesser extent into the rest of the respiratory tract and the lungs. The distribution of the inoculum is influenced by the volume used for the challenge. Furthermore, the volume used to inoculate mice correlates directly with the severity of the infection. Haste *et al.* reported that the survival of MF1 mice was approximately 20% when infected with $1x10^6$ CFUs of the D39 strain in 40 or 50 µL of inoculum, compared to approximately 80% survival when an inoculation volume of 20 µL was used [430]. In paper I, II and III an infection volume of 20 µL was used, and the infection dose was $5x10^6$ CFUs in paper I and III and $1x10^7$ CFUs in paper II. By intratracheal instillation of the inoculum into the trachea the nasopharynx is bypassed allowing for 99% inoculum to reach the lower respiratory tract and lungs directly [431].

Systemic disease is mimicked by challenging the mice by injecting the inoculum into the intraperitoneal cavity or directly into the circulatory system. Intraperitoneal infection often results in a more rapid progression than intravenous infection [432]. The intraperitoneal cavity is not an anatomical site where pneumococci are found normally. For this reason, the papers included in this thesis mostly made use of the intravenous infection route for the characterization of virulence during invasive disease.

Passaging of bacteria prior to use for in vivo infection experiment is a method often used in publications as it increases the virulence and homogeny of the inoculum. It has been long known that comparatively few passages *in vivo* can increase the virulence of isolates after extended culturing *in vitro* [433]. Pandya *et al.* showed that culturing the TIGR4 strain on blood agar for 50 or 100 passages reduced the expression level of several virulence genes, but this did not correlate with reduced virulence in mice after intraperitoneal infection [434]. The

passaging is carried out by injecting bacteria into the peritoneal cavity, bacteria are then isolated from blood following dissemination. To increase the yield the passaged bacteria are then cultured *in vitro* before use in infection experiments [306]. The papers included in this thesis characterize the virulence of clinical isolates, as passaging introduces bottlenecks it may introduce a virulence profile not present in the isolated population. Hence, the practice of passaging the inoculum has not been used in papers I-IV.

3.1.8.5 Measurements of lung function

The physiological measurements of lung function were performed using Forced Maneuvers system according to an established model [430]. There are only a few reports on the effects of pneumococcal infection on lung function of mice [430, 435]. Chronic infection with *S. pneumoniae* led to a reduction of the forced expiratory volume (FEV) at 7, 14 and 21 days post infection, but no effect was seen at 24 hours post infection [430]. The FEV indicates limitation of the airflow in the airways which can be caused by inflammation of the airways.

The residual volume (RV) is the volume of air left in the lungs after full expiration. The functional respiratory capacity (FRC) includes the RV but also includes the expiratory reserve volume (ERV), which is the volume of air that can be expelled after exhalation at rest. Both RV and FRC are reduced by edema in the lungs, as this reduces the volume of air that lungs can accommodate.

3.1.9 Ethical considerations

3.1.9.1 Patient information

The collection of strains for paper I had been approved by the local ethical committee prior to the start of the study.

3.1.9.2 The use of animals in research

Animals were kept in accordance with the requirements of the ethical permits and the animal facilities at Karolinska Institutet. The conditions include 12 hour dark/light cycles and access to nesting material and food and water *ad libitum*. All experimental procedures performed in papers I, II, III and IV were approved by the local ethical committee "Stockholms Norra Djurförsöksetiska Nämnd". Following experimental procedures the "Assessment of health conditions of small rodents and rabbits when illness is suspected" scoring template was used (Figure 8).

2008-03-17

Karolinska Institutet

Assessment of health conditions of small rodents and rabbits when illness is suspected

Observations	Points	Ref. value	Observations, explanations
General condition		0.0	Awake, active, reacts to stimulation
		0.1	Burrows in litter, hides, lies still but is startled when touched
		0.4	Immobile, little or no voluntary movement, burrows/hides, presses head against cage bottom, vocalizes, extremely afraid and/or aggressive when touched
Porphyria*1 (not rabbits) and/or eye inflammation		0.0	No discoloration, clear and clean eyes
		0.1	Some porphyria and/or discharge around eyes and nose (can be difficult to see in colored animals)
		0.4	Obvious porphyria in "face" and/or on legs and paws, squints and/or discharge around eyes
Movements and posture		0.0	Normal
		0.1	Moderate incoordination*2 when animal is stimulated; hunched posture
		0.4	Marked incoordination, head held at angle, hunched posture and/or back, do not supports itself on all four limbs, and/or paralysis
Piloerection 3*		0.0	Fur smooth and well-groomed
		0.1	Moderate piloerection
		0.4	Severe piloerection, sticky and ungroomed fur
Respiration		0.0	Normal respiration
		0.4	Breathes with open mouth, abdominal breathing or panting, rales and/or gasping
Skin		0.0	Skin covered with fur and without sores or other signs of injury
		0.1	Small sores or scabs without infection; itching
		0.4	Bites or scratches itself or others inducing sores and/or signs of infection such as redness, pus formation or oozing; sticky and ungroomed fur. Operation wounds that do not heal, or broken sutures
Observations			Observations, explanations
Total points			***Notes***

1*porphyria= red-colored secretion from eyes (or nose)

2* Unsteady, has difficulty coordinating movements

3* Hairs of fur appear harsh because of partial erection, animal appears to be cold

When point total is higher than 0.3, the animal is to be euthanized or the veterinarian is to be contacted.

Figure 8. Scoring template used at Karolinska Institutet for assessing the health of rodents following *in vivo* procedures. This scoring system has been used in papers I-IV, in conjunction with the ethical permits. The maximum score allowed during a procedure is dictated by the ethical permit and may deviate from the guideline in the scoring template.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Intraclonal variations among *Streptococcus pneumoniae* isolates influence the likelihood of invasive disease in children.

Paper I is an epidemiological survey of pneumococcal serotypes and clonal types in the region of Stockholm between 1997 and 2004. It is also an investigation of the invasive disease potential of serotypes and clonal types.

In total 715 pneumococcal isolates were collected from children under the age of 18 years, in the Stockholm area. Of these isolates 550 were obtained from carriage in healthy individuals and 165 were isolated from cases of invasive disease. Serotyping and PFGE were performed on all isolates to determine the serotype- and clonal distribution of the strains. Two hundred isolates, including all invasive isolates and 35 carriage isolates, were further analyzed with MLST to determine the ST.

The analysis of the PFGE patterns made it possible to further discriminate between genetic lineages within serotypes and STs. PFGE analysis revealed three major PFGE clones within the CC138 lineage of serotype 6B. These PFGE clones were associated with different potential for causing invasive disease. Subclone SWE6B-3 had a high OR for invasive disease, while the subclones SWE6B-1 and SWE6B-2 had lower ORs. Four isolates were selected from the three PFGE clones and were whole-genome sequenced. These were the invasive isolate BHN237 and the carriage isolate BHN427 from subclone SWE6B-1, the carriage isolate BHN418 from subclone SWE6B-2, and the invasive isolate BHN191 from clone SWE6B-3.

Sequence analysis of the strains revealed that the isolates were genetically similar. However, they differed in the distribution of prophages. It has previously been shown that prophages can affect the PFGE pattern [436]. BHN418 was the only isolate of the four that lacked an integrated prophage, together with 74% of the other 6B strains belonging to SWE6B-2. In comparison all isolates of SWE6B-3 harbored at least one prophage, and the corresponding percentage for SWE6B-1 was 95%. In clones SWE6B-2 and SWE6B-3 the prophage group 3 predominated.

Moreover there were some differences in insertions and deletions in the genomes. BHN237 and BHN427 encoded accessory region 29, including a partial ABC transporter. The accessory region 13 was present in BHN191 and BHN418, and encodes a putative restriction system. Two open reading frames replaced the virulence factor PcpA in the carriage isolate BHN417. There were also allelic variations for the virulence factors PspA and PspC. Isolate

BHN191 showed much greater ability to bind human factor H compared to the other sequenced strains despite having the same allelic variants of PspC, PspC6.9 and PspC9.4, as strain BHN418. Strains BHN237 and BHN427 had PspC6.1 and PspC9.1 alleles.

We show that the invasive disease potential in children may differ between clonal types, but can also vary within clonal types as determined by PFGE. The study confirms the serotype-oriented paradigm in virulence, as it shows that serotype is important as a virulence determinant, illustrated by the high ORs seen for serotype 1, 7F and 14. These serotypes are often found to be associated with invasive disease [437-439].

One aspect of the study is that it stresses the influence of the clonal lineage and shows the importance of the genetic context outside the capsule locus. The results from this study show differences in the invasive disease potential between clonal lineages within serotype 6B. This is in line with previous studies where lineages have been associated with invasive disease, although not independently of serotype [437]. In an era when vaccination selects against the serotypes currently associated with invasive disease, it is likely that lesser serotypes find a niche to exploit. These emerging capsular types may not necessarily have the same potential to cause invasive disease as the capsules of serotypes 1, 7F and 14. In a scenario where an increasing number of strains carry capsular variants with similar contribution to virulence, the genetic context outside the capsule locus becomes ever more important for epidemiological surveys and in vaccine development.

In the context of vaccine development the study reveals that there can be a considerable genetic variation within a genetic lineage, and that this extends to potential vaccine candidates such as PcpA, PspA and PspC [266, 440-442]. As these proteins are described as virulence factors the variability between isolates could influence the virulence of the strains. PcpA was not present in the carriage strain BHN427 and this strain was attenuated in the mouse model after intranasal challenge (Figure 3A in paper I). However, we did not see a difference in the ability of the strains to colonize mice (Figure 9A), which is in line with a dispensable role of PcpA in colonization [443-445]. The data does not support a previously described role for PcpA in adherence to human nasopharyngeal cells [445]. The result is consistent with a role in adherence as described for human type II lung epithelial cells, and for the involvement of PcpA in the establishment of pneumonia in mice [443, 444]. There was no difference in the level of bacteremia between the strains after intravenous infection (Figure 9B), which suggests that PcpA exerts its function in the respiratory tract.

The *pspA* clade was in contrast to a previous publication not associated with the clone [446], which is consistent with the study by Qian *et al*. [447]. Interestingly the invasive isolate BHN191 showed much greater binding of factor H than the other three strains of the same serotype and clonal complex. The increased binding of factor H could potentially influence its ability to cause invasive disease. Serotype, capsule amount and PspC are factors known to

affect the binding of factor H [185, 256, 448]. The carriage isolate BHN418 had the same *pspC* allele as BHN191.

In this study we conclude that clonal type as well as intra-clonal variation affects the invasive disease potential in children. This study underlines the plasticity in the pneumococcus and the complexity in designing protein-based vaccines against pneumococcal lineages. We also show that there is marked variability in the ability to bind factor H and this suggests a difference in the selective pressure exerted by the complement system on different variants of the same clonal lineage.

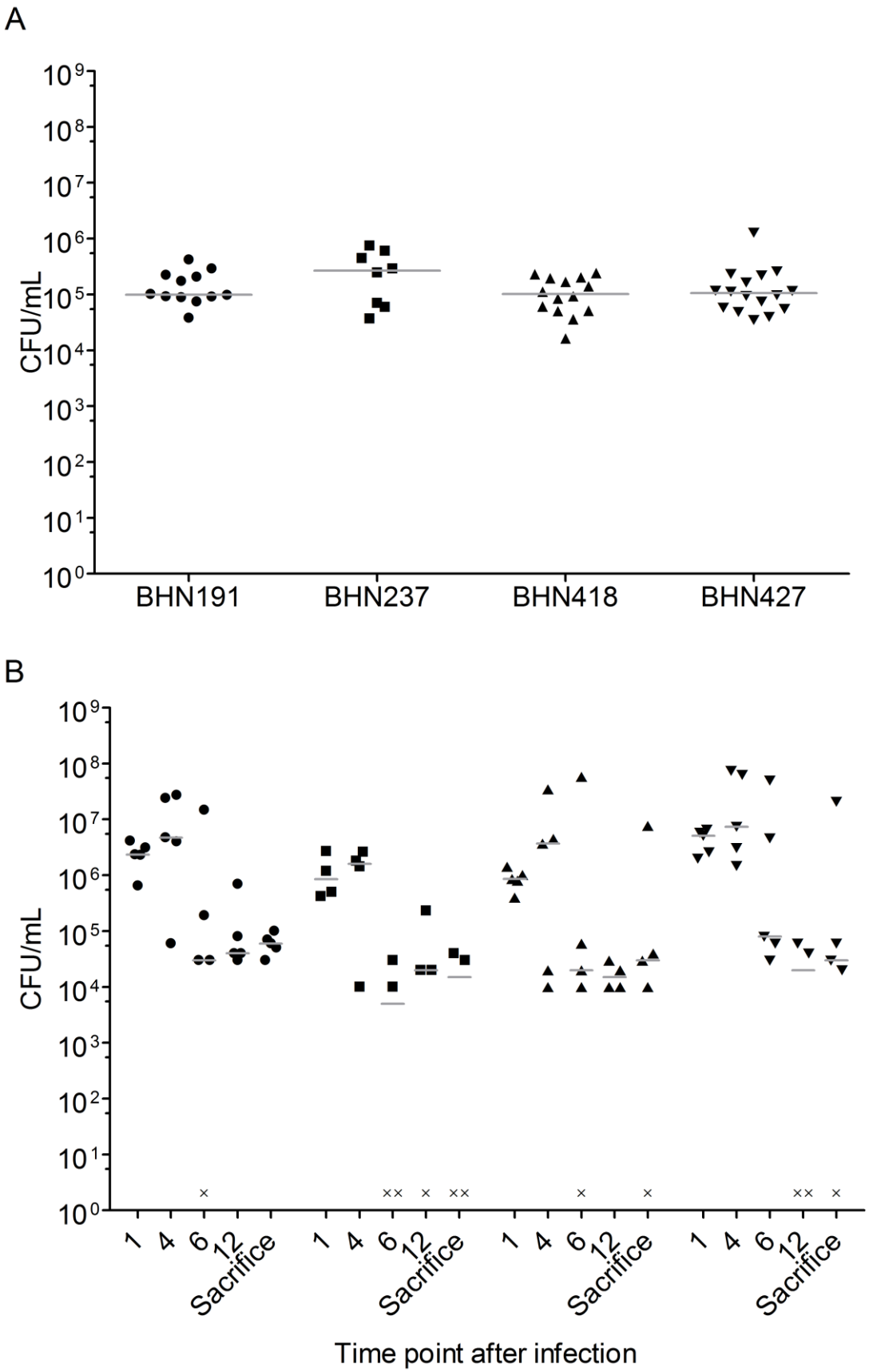

Figure 9. Challenge of 7-8 weeks old C57BL/6 mice. Nasopharyngeal lavage of healthy survivors 168 hours after intranasal infection with $5x10^6$ CFUs (A). Level of bacteremia during the 24 hours following intravenous infection with $5x10^6$ CFUs (B). BHN191 (●), BHN237, (■) BHN418(▲), BHN427 (▼), values below the detection limit are denoted with (×). There were no significant differences between groups in colonization (A) or between corresponding time points (hours) after intravenous challenge (B).

4.2 PAPER II

Emergence of hypervirulent mutants resistant to early clearance during systemic serotype 1 pneumococcal infection in mice and humans.

Paper II started with the observation of aberrant colonies, morphologically different from the wild-type serotype 1 strain, appeared 24-48 hours after intraperitoneal challenge in mice. The mutant variant formed bigger colonies than the wild-type. Upon infection with the big morphotype the infection progressed much faster. The causative mutations were found in the *spxB* gene, encoding the enzyme pyruvate oxidase. The mutations resulted in an almost complete inability to produce hydrogen peroxide, and complementation of *spxB* restored hydrogen peroxide production and resulted in virulence similar to that of the wild-type strain. The production of hydrogen peroxide is an important virulence factor for pneumococci as it allows the bacterium to compete with other bacterial species in the nasopharynx [372]. In this study we were able to show that impairment in the hydrogen peroxide production leads to altered characteristics of the bacterium, which results in a more virulent phenotype during invasive disease.

Most of the characterization in paper II is done with the BHN682 strain, a wild-type isolate with a small morphotype and normal hydrogen peroxide production, and the mutant BHN683 strain with a big colony morphotype and impaired hydrogen peroxide production. These isolates were recovered from a patient with invasive disease, proving that the hyper-virulent variant population arises during infections in humans and could be of clinical importance.

The big variant was more virulent than the wild-type in after both intranasal and intravenous challenge. In mice challenged intravenously with an inoculum consisting of a mix of the bacterial wild-type and big morphotype, the two bacterial populations behaved similar to what was seen in mice infected with only one of the strains. This suggests that there is no synergism between the two bacterial populations as they behave as distinct populations. It argues for that the difference seen between the populations is intrinsic to the ability of the bacterium to cope with the environment of the host. Thus the clearance of the wild-type bacteria does not seem to be caused by a more potent activation of the host immune response to the infection.

The increase in virulence of the big morphotype correlated with an inability of the host to clear the bacteria during the initial stage of infection. Furthermore, we could show that macrophages were important in the differential clearance of the two variants, as the hyper-virulent mutant was less phagocytosed *in vitro*. Injection of liposome-encapsulated clodronate leads to the depletion of macrophages in the spleen. Depletion of macrophages resulted in a rapid increase in bacterial numbers in mice infected with the wild-type moprhotype, similar to what was seen for mice infected with the mutant strain. Mice lacking SIGN-R1 expressed by marginal zone macrophages showed decreased ability to clear infections with the wild-

type strains. In contrast no contribution of SIGN-R1 was seen to the clearance of the mutant strain. We did not detect a significant contribution made by neutrophils in the clearance of either strain.

The capsule is required for systemic infections and its expression is upregulated during these conditions [227]. Hence, differential capsule expression could offer a potential explanation for the difference in clearance mediated by splenic macrophages, which was observed for the two strains. Furthermore the level of encapsulation affects the ability of macrophages to phagocytose the bacteria [449]. However, we could not detect a significant difference in the degree of encapsulation between the wild-type and the mutant strains.

SpxB encoded hydrogen peroxidase converts pyruvate to acetyl-phosphate during micro-aerobic conditions generating hydrogen peroxide in the process. Acetyl-phosphate can then be converted to acetyl-CoA or to acetate in subsequent enzymatic reactions [450]. As acetyl phosphate plays an important role in the metabolism of pneumococci it would be conceivable that perturbation in the pathways of pyruvate utilization, by mutations in *spxB*, could have far-reaching consequences for the expression profile of the bacterium. It has been shown that the deletion of *spxB* led to reduced adherence to human type II lung epithelial cells and endothelial cells, and this phenotype could be restored by addition of acetate [450].

Carvalho *et al.* showed that mutations in the *spxB* gene lead to minor changes in the gene expression of the bacterium during micro-aerobic *in vitro* conditions, arguing against large-scale changes [230]. The authors did note a 30% increase in the amount of capsule, but no increase in the gene expression of the capsule locus could be seen [230]. However, this study was made with the D39 strain. When we deleted the *spxB* gene in the TIGR4 strain we did not see a difference in the survival, and only minor differences in the level of bacteremia. In addition there was no pronounced early clearance seen for the TIGR4 wild-type strain. It is therefore possible that the aggravation of the infection seen for the *spxB*-mutants in serotype 1 is not a general phenomenon amongst pneumococcal serotypes. In line with this view, Orihuela *et al*. showed that deletion of *spxB* in D39 led to a minor reduction in bacteremia compared to the parental strain [322].

In this study we show the importance of SIGN-R1 in the clearance of the wild-type strain. This finding is consistent with the described importance for this receptor in mediating phagocytosis and activation of complement [133, 135, 137]. The importance of the spleen in the protection against pneumococcal bacteremia has been described in animal models previously [451-453]. Similarly, splenectomy is an important risk factor in humans for developing pneumococcal bacteremia [454].

It is not clear how the inactivation of the *spxB* gene results in the ability to evade the immune system. It is likely that the effect is pleiotropic, which can relate into changes in metabolism and consequently changes in regulatory pathways. Changes in the capsule could also

contribute to increased virulence, and potentially the absent effect of hydrogen peroxide itself as well. From a biological standpoint paper II presents an interesting example of an ongoing selection during the course of an infection. It illustrates that during the course of an infection a bacterial population can diversify, generating variants with different abilities to cause disease.

4.3 PAPER III

***Streptococcus pneumoniae* strains with similar mortality show different patterns of disease progression in mice.**

The manifestations of severe pneumococcal infections include lobular pneumonia with tissue consolidation and septicemia. In paper III we characterize the pathology and progression of two pneumococcal strains which show different propensities to cause either pneumonia or bacteremia.

The two strains, Sp3-BS71 of serotype 3 and D39 of serotype 2, were highly virulent in mice after intranasal infection with approximately 30% survival. The median time to sacrifice was 37 hours post infection for serotype 2 and 35.5 hours for serotype 3. The bacterial load in the lungs was comparable for the mice that succumbed following intranasal challenge. A striking difference was observed in the dynamics of the bacterial clearance from the lumen of the lungs. While the number of serotype 3 bacteria remained stable for 24 hours, a large proportion of the serotype 2 bacteria were cleared from the lungs. This reduction is in agreement with a previous study in which infection with serotype 2 resulted in a transient decline in bacterial numbers, while the reduction was less pronounced for serotype 3 [364]. We could show that murine macrophages *in vitro* had an increased potential to bind serotype 2 than serotype 3 pneumococci, and that they were unable to phagocytose the latter. We also showed that serotype 3 bacteria had decreased adherence to both human nasopharyngeal cells and type II lung epithelial cells.

To explore the importance of cells expressing CD11c we used a transgenic mouse model in which the simian diphtheria toxin receptor is coupled to expression of CD11c. The model allows for ablation of CD11c-expressing cells by administering diphtheria toxin. There was a difference in the disease progression in mice lacking CD11c-expressing cells compared to wild-type mice. However, we noticed that treatment with diphtheria toxin in CD11c-transgenic mice was associated with neutrophilia, which has been described by others as well [420, 421]. The neutrophilia om CD11c-transgenic mice caused a reduction in the bacterial load in the lungs of mice infected with the serotype 2 strain, as well as a reduced bacteremia. This is consistent with a well-established role of neutrophils in clearing pneumococci in the respiratory tract [146, 429, 455]. Depletion of neutrophils in wild-type mice did not increase the bacterial load in lungs or cause increased dissemination compared to untreated mice, which is consistent with neutrophils infiltrating after the initial response by alveolar macrophages [456].

The difference in the cytokine response to the two strains was modest, but the elevated pro-inflammatory response at 24 hours post infection for the serotype 3 was consistent for several cytokines. The higher bacterial load in the lungs 24 hours after infection and the increased levels of pro-inflammatory cytokines corresponded with a reduced lung capacity of mice

infected with serotype 3. Mice infected with serotype 3 also had a focal pattern of inflammation during pneumonia, with local consolidation of the lung tissue. The inflammation caused by serotype 2 was more diffuse. Although depletion of alveolar macrophages reduced the clearance of both strains in the lungs this did not lead to an increase in bacteremia. A possible interpretation of this finding is that macrophages play a key role in controlling bacterial numbers early in pneumonia but also that the ability to disseminate into blood is in part determined by other factors. The reduced clearance of bacteria in the lungs of mice depleted of macrophages is in line previous results, which argue that macrophage-dependent clearance can be saturated by high inoculums [449, 456].

In a previous publication Kelly *et al.* found that expression of the serotype 3 capsule in other serotypes could render virulent strains avirulent as well as increase the virulence of other strains [457]. However, no difference was seen in the virulence for D39 expressing the serotype 3 capsule, compared to the parental D39 strain. The co-adaptation of the functions of the capsule and virulence factors has also been suggested in a study by Kadioglu *et al.* where FP50 the same capsule switch strain as in paper III was used [364]. The FP50 strain showed a reduced ability to colonize the nasopharynx compared to D39 and was in this respect similar to the serotype 3 strain. In the lung the bacterial load for the serotype 3 strain was mirrored by FP50 during the initial hours of infection, after which FP50 showed similarity with D39. In the end FP50 was attenuated with respect to both serotype 3 and D39 [364]. This suggests that the relative contribution of capsule compared to other virulence factors vary during the course of an infection.

A potential explanation for why the serotype 3 strain was less affected by clearance mediated by phagocytic cells was found in the ability to avoid C3-deposition. C3-binding to the serotype 3 strain was much lower than the binding to the serotype 2 strain or the FP50 strain. That the FP50 mutant showed C3-binding more similar to the serotype 2 than the serotype 3 strain was somewhat surprising as capsule has a big influence on C3-deposition [458]. It has previously been shown that C3-deposition can be reduced by introducing the serotype 3 capsule into a serotype 2 (D39) strain [459]. However, it is difficult to separate the contribution of capsule from that of other genetic factors as Hyams *et al.* reported higher C3-deposition on the TIGR4 strain expressing the serotype 3 capsule than the serotype 2 capsule [185]. Using different serotypes Youste *et al*. reported approximately three times higher deposition of C3 for serotype 3 than for D39 [280].

The pneumococcal virulence factor PspC binds human factor H, but is unable to bind murine factor H. Our sequence analysis of the *pspC* genes revealed that the serotype 3 strain carried a version of *pspC* with a factor H binding motif more similar to that of the serotype 1 strain ATC33400, than that of other serotype 3 strains such as A66, or to the serotype 2 strain D39. It is not known if these variations in the binding site would translate into a difference in the ability to bind factor H. Regardless of the influence of PspC on binding of human factor H, the reduced deposition of murine C3 seen for the serotype 3 strains must be explained by

means other than binding of factor H. It is possible that the different variants of PspA affect the complement deposition, but previous studies have shown that the different families have similar contribution to virulence in mice [253].

In several studies it has been suggested that the capsule and genetic background is interlinked and that not all capsules function in certain genetic backgrounds [280, 364, 457, 459]. In line with these earlier reports, the protection against C3-deposition observed in paper III cannot solely be attributed to the properties of the capsule or a particular virulence factor. The striking difference in C3-deposition in both mouse and human serum is intriguing and warrants further investigation.

4.4 PAPER IV

A bacteriophage contributes to virulence of *Streptococcus pneumoniae* serotype 1 during invasive disease.

Temperate bacteriophages are frequently found within the genomes of clinical isolates of *S. pneumoniae*. Despite of intact and remnant prophages being common, little is known about their effect on virulence of the bacterium which harbors them. In pneumococci the lysogenic phage MMI has been shown to increase adherence to human pharyngeal cells and aid in the colonization of mice [460]. However, a more recent study observed a fitness defect of the strains during colonization and hypothesized that this might be compensated for by an increased tolerance to penicillin [461]. Paper IV describes the contribution of a prophage to the virulence of the wild-type serotype 1 strain used in paper II.

Despite being poorly transformable we were able to delete the whole phage from the serotype 1 strain. In an intravenous infection model we observed a marked delay of severe disease for the mutant serotype 1 strain lacking the prophage. This was reflected in a reduced level of bacteremia compared to the wild-type during the first 24 hours of infection, which persisted until the animals were euthanized.

It has previously been reported that a similar prophage in the closely related species *Streptococcus mitis* influenced the ability of *S. mitis* to bind to platelets [462]. The binding to platelets was mediated by two proteins PblA and PblB [462-464]. Subsequently a pore-forming phage-encoded holin as well as the phage endolysin was shown to be required for surface localization of PblA and PblB [465]. The holin and endolysin may therefore be required for PblA and PblB to function properly by permitting the surface localization of these proteins. It would also be conceivable that the holin and endolysin could have functions analogous to LytA, the major autolysin in pneumococci. Furthermore, *in vitro* studies show that the endolysin binds to fibrinogen and is a virulence factor during systemic infections of lagomorphs [466].

In order to determine which part of the phage contributed to the virulence in pneumococci, mutants lacking these putative virulence genes were generated and used to challenge mice. Surprisingly, all mutants showed a decreased virulence resulting in significantly longer survival time than for mice infected with the wild-type strain carrying the intact phage. However, only for constructs lacking PblB was the increased survival time reflected in reduced bacterial load in blood. It seems likely that PblB confers the increased ability for the pneumococcus to survive in blood, absent in the construct lacking the whole phage. It is therefore unlikely that the holins and endolysin are required for surface localization of PblB in *S. pneumoniae.* It was very recently shown by Hsieh *et al.* that PblB mediates binding to platelets and type II lung epithelial cells in a serotype 14 strain [467]. The authors could also show that the wild-type strain outcompeted a PblB deficient derivative in the nasopharynx

and lungs of infected mice [467]. However, the role of PblB during systemic infection was not evaluated.

In conclusion, the *pblB* gene carried by a prophage contributes to the virulence of serotype 1 after intravenous challenge. This conclusion is consistent with a previous study by Harvey *et al*., where the authors observed that a genetic region containing a similar prophage was associated with increased virulence after intraperitoneal and intranasal infection of mice [468]. The genetic region affected the bacterial burden during pneumonia and bacteremia but not the bacteria's ability to colonize. In *S. mitis* PblA and PblB contributed to the virulence in a lagomorph endocarditis model [465].

As attempts to complement the deletions were unsuccessful it has not been possible to investigate the change in maximum optical density during growth *in vitro*. All mutants with partial deletions of the phage grew to higher optical densities than the wild-type strain or the mutant lacking the whole phage.

5 CONCLUDING REMARKS

Streptococcus pneumoniae causes considerable mortality and morbidity worldwide. Pneumococcal disease strikes hardest against young children in low- and middle-income countries, but the human suffering caused by pneumococcal disease and the cost associated with caring for those affected is a burden shared by all societies.

The nature of the human interaction with the pneumococcus is complex. On one hand the pneumococcus is a devastating pathogen, but it is also a common colonizer frequently carried throughout a human lifetime without causing any symptoms. A bacterial infection can often be serious, and we depend on our immune system to keep us healthy, but much of the pathology associated with pneumococcal pneumonia is caused by the response of the immune system. The role of inflammation is even more evident during the most severe of pneumococcal diseases, meningitis, as the inflammatory response causes most of the pathology.

The ability of the pneumococcus to differentiate in order to adapt to its environment is one of the most intriguing aspects of pneumococcal biology. As the pneumococcal pan-genome contains a plethora of virulence factors, of which a given strain expresses only a selection, it is difficult to find causality between genotype and pathogenicity. The genetic mosaic is constantly being remodeled by capsule switches, natural transformation, rearrangement and gene transfers caused by bacteriophages and spontaneous mutations. The genetic flexibility gives the pneumococcus the necessary adaptability to survive, and is a source of admiration, as well as frustration, to those who wish to study its biology.

In paper I we show that even within an epidemiologic clone there are genetic variants with different potential to cause disease. The variants also exhibited considerable differences in genetic content and variability in virulence genes. These genetic variations translated into a clear difference in the interaction with factor H, which is important for complement evasion.

In paper II we show that selection for different populations occurs during the course of an infection, and that these populations can have different virulence potential. We show the emergence of hyper-virulent mutants that are deficient in the *spxB* gene during systemic infection. The *spxB* gene increases the fitness during colonization is not favored during systemic disease. We also show the importance of splenic macrophages in clearing bacteremia. The results from this paper emphasize the need to investigate population dynamics of bacterial infections.

All pneumococci are not equal in their ability to cause pneumonia and invasive disease, a difference which is made clear in paper III. We show that despite a fairly effective immune response to serotype 2 that reduced the bacterial burden in the lungs, the infection

disseminated rapidly. In contrast serotype 3 largely persisted in the lungs. but did not readily disseminate. We also show a clear difference in the ability of complement to opsonize the two strains, and a difference in the role of phagocytic immune cells in controlling the infection.

The contribution of phages to the virulence properties of the pneumococcus is poorly understood. In paper IV we show that a bacteriophage contributes to the virulence during systemic infection via the gene encoding the tail protein PblB. The presence of the *pblB* gene reduces the ability of the host to clear bacteria from the bloodstream, but through what function *pblB* confers this effect remains unknown.

The adaptability of the pneumococcus will keep it an important pathogen for the foreseeable future. However, the encouraging results of the vaccine introduction show that we can reduce the impact of the most severe serotypes and also the burden of pneumococcal disease. If the new situation will consist of a greater number of serotypes with similar contribution to disease, it may not be feasible to continuously expand the valency of serotype-based vaccines. Vaccines targeting the virulence factors of the predominant genetic lineages associated with disease should therefore be developed to complement the current strategy.

Taken together the data presented in this thesis stress the complexity of pneumococcal world, "the pneumoverse". We are now on the brink of being able to use genomic and transcriptional sequencing to ask questions on the dynamics of bacterial populations during the course of an infection. The immune system constantly selects against the bacterial variants with the lowest fitness. In addition there are successive bottlenecks imposed on the bacterium as it transverse barriers and move between environments. These selective forces undoubtedly shape the genetic and transcriptional profile of the population, by favoring the best adapted variants and by chance. As we increasingly are being able to study these differences between bacteria during the course of an infection, we can start to elucidate the selective pressures which confer a selective advantage to these changes at various steps of the infection process. As suggested by the finding in paper II it may be that bacterial subpopulations affect the pathology and outcome of the disease than previously thought.

The complexity of pneumococcal pathogenesis is greater than what can be understood from studying the handful of strains that are commonly used. Although these strains makes it possible to study processes and factors in great detail, they will not allow for the vast variety and combinatorial effects present in the pneumococcus to be taken into account. It is in this respect that the characterization of clinical isolates gives new insights and relevance to pneumococcal biology.

6 RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

ORRSKOG, S., ROUNIOJA, S., SPADAFINA, T., GALLOTTA, M., **NORMAN**, **M**., HENTRICH, K., FALKER, S., YGBERG-ERIKSSON, S., HASENBERG, M., JOHANSSON, B., UOTILA, L.M., GAHMBERG, C.G., BAROCCHI, M., GUNZER, M., NORMARK, S., HENRIQUES-NORMARK, B., 2012. Pilus adhesin RrgA interacts with complement receptor 3, thereby affecting macrophage function and systemic pneumococcal disease. MBio 4, e00535-00512.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Syftet med denna doktorsavhandling var att studera förloppet under det tidiga skedet av infektioner orsakade av *Streptococcus pneumoniae*, en bakterie som även kallas pneumokocker. På förskolor bär omkring 60 % av barnen på pneumokocker i näsan utan att utveckla sjukdom, så kallat asymptomatiskt bärarskap. Samtidigt dör årligen omkring 700,000 barn under fem år i världen av lunginflammation eller invasiva sjukdomar som blodförgiftning och hjärnhinneinflammation orsakade av pneumokocker. Pneumokocker kan grovt indelas i 97 olika varianter, så kallade serotyper, beroende på sammansättningen hos den kapsel som täcker bakteriernas yta. Kapseln är av stor betydelse för pneumokockernas förmåga att orsaka sjukdom då den förhindrar att immunceller såsom makrofager och neutrofiler tar upp och dödar bakterierna. Utöver serotyper skiljer sig olika stammar av pneumokocker även med avseende på den arvsmassa de bär i form av DNA, därigenom skiljer de sig även i vilka funktionella molekyler som de kan uttrycka. Eftersom uppsättningen av de olika funktionella molekylerna, oftast proteiner, varierar mellan olika bakteriestammar så skiljer sig även stammarnas förmåga och tillvägagångssätt som de orsakar sjukdom på.

De delarbeten som presenteras i denna avhandling avsåg att öka vår kunskap om hur pneumokocker orsakar sjukdom. Därför jämfördes hur olika bakterier som isolerats från patienter eller bärare skiljer sig åt med avseende på deras förmåga att orsaka sjukdom.

I avhandlingens första artikel jämfördes fördelningen av serotyper funna hos barn som var bärare respektive hade invasiv sjukdom. I likhet med tidigare studier visade resultatet att vissa serotyper var mer benägna att orsaka att sjukdom än andra. Studien kunde även påvisa att genetiskt närbesläktade pneumokocker kan skilja sig i benägenhet att orsaka invasiv sjukdom. När dessa närbesläktade stammar karaktäriserades ytterligare visade det sig att en invasiv variant hade en ökad förmåga att binda faktor H. Genom att binda faktor H kan pneumokocken inaktivera komplementproteinet C3b som också binder till bakteriens yta. Eftersom aktivt C3b gör det lättare för makrofager och neutrofiler att ta upp och avdöda bakterien, så blir pneumokocken mer motståndskraftig mot denna del immunförsvaret genom att inaktivera C3b.

I det andra delarbetet påvisades att det under sjukdomsförloppet uppkommer varianter av pneumokocker som är mer sjukdomsframkallande än den ursprungliga stammen. Dessa varianter saknade en funktionell gen som ger bakterien bättre möjlighet att kolonisera bärare. Resultaten visade att de bakterier som saknar denna funktion blir mer motståndskraftiga mot immunsvaret vid blodinfektioner, genom att de inte lika lätt tas upp av makrofager i mjälten.

I det tredje delarbetet undersöktes två bakteriestammar med olika serotyp, där den ena orsakade en hög grad av lunginflammation, medan den andra hade en större benägenhet att orsaka invasiv sjukdom men var sämre på att överleva i lungorna. Makrofager i lungorna

spelade en stor roll då de hade förmåga att avdöda den ena stammen pneumokocker, som ändå lyckades orsaka sjukdom genom att sprida sig till blodomloppet. Den andra stammen av pneumokocker var däremot väldigt motståndskraftig mot makrofager, troligen berodde detta på att komplementproteinet C3b inte lyckades binda till bakteriens yta.

Bakterier kan bli infekterade av bakteriella virus, så kallade fager, som kan använda bakterien för att föröka sig och sedan spridas vidare genom att döda bakterien. Andra typer av virus, så kallade profager, integreras istället i bakteriens arvsmassa och sprids tillsammans med sin värds arvsmassa då bakterien delar sig. Det är känt att profager är vanligt förekommande bland pneumokocker, men väldigt lite är känt om hur detta påverkar bakteriens förmåga att orsaka sjukdom. I det fjärde delarbetet visades att om en profag togs bort ur arvsmassan hos en mycket virulent stam av pneumokocker, förlorade bakterien sin förmåga att överleva i blod.

Sammantaget påvisar avhandlingen betydelsen av den stora variation som finns hos pneumokocker, och som påverkar deras förmåga att orsaka sjukdom. Det är genom att förstå och fördjupa vår kunskap om de olika tillvägagångsätt som finns hos denna mångfacetterade bakterie som vi kan förstå dess livsbetingelser. I förlängningen är det genom ökad kunskap om de grundläggande förutsättningarna som styr pneumokocker som leder till att vi på bättre sätt kan förhindra och även behandla de olika sjukdomar som pneumokocker ger upphov till.

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I will conclude this thesis with two numbers that have shaped me as a person during this period of my life, 2,369 and 2,172. I should remember them both.

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